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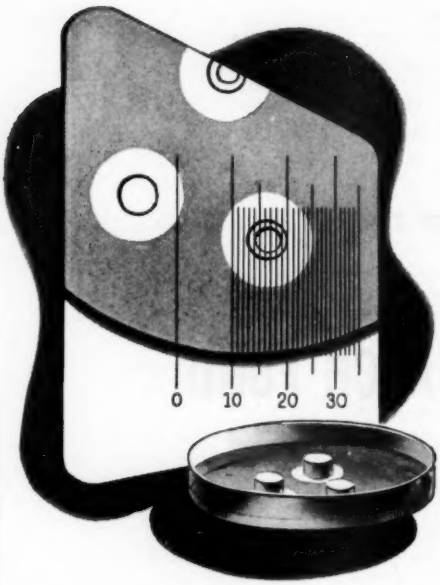

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A Study of an Encephalitic Strain of Hog Cholera Virus

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East Lansing, Michigan

A SOMEWHAT CYCLICAL plague, hog cholera has exacted severe losses in the United States since it was first recognized in 1833 in Ohio. The annual loss from hog cholera, estimated at 30 to 40 million dollars, emphasizes the problems still to be solved in controlling this disease. Postvaccination swine troubles, which were a major problem in 1949 and 1950, have figured noticeably in the annual loss from hog cholera. Considerable evidence has been presented to show that a variant strain of hog cholera virus has been the cause of many of the vaccination failures. Difficulty in maintaining the unstable variant in serial animal passage during serum production explained the lack of protection given by serums which later demonstrated low potency against the variant. In view of these factors, the investigation reported in this paper was instigated to study an atypical strain of hog cholera virus, to determine how this virus differs from the more typical strains and how to maintain these atypical characteristics in serial passage.

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Dr. Dunne is a former Fellow of the American Veterinary Medical Association, assigned to the Department of Animal Pathology. This paper was submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in animal pathology in the School of Graduate Studies, Michigan State College, by Dr. Dunne.

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REVIEW OF LITERATURE

The stability of hog cholera virus in terms of virulence or as to antigenicity has been the subject of much speculation. Hupbauer[1] tested strains of hog cholera virus from the United States, Argentina, and Spain against serum of German origin and concluded that plurality of types does not exist in the sense that types exist in foot-and-mouth disease. Köbe and Schmidt[2] were able to produce a chronic type of hog cholera by dilution of the virus and concluded that chronic hog cholera was not the manifestation of a type of virus but rather a question of reduced virulence, and associated with the amount of the infecting dose of virus. Kernkamp,[3] however, cited specific field cases of chronic hog cholera with seventeen-day contact incubation periods occurring in vaccinated as well as nonvaccinated droves.

A virus of an entirely different antigenic nature was reported by Montgomery[4] in British East Africa and by De Kock[5] *et al.* in South Africa. The disease produced by this virus followed the same pattern of symptoms and lesions as the European and American forms of hog cholera but had a short incubation period of thirty-six hours to four days, with the average being about two days from the time of injection.

The vaccination disasters of 1949 and 1950 resulted in investigations by Dale *et al.*,[6] who showed definite indications of a variant nature of the virus involved. Of foremost interest in their work was the failure of some commercial serums to protect against certain viruses isolated from field cases. No mention of nervous disorders was made in the paper, although apparently such have been common in a number of so-called vaccination "breaks." An editor's note[7] stated that "outbreaks of unidentified nervous disorders in swine have been reported by practitioners in widely separated

locations in the cornbelt." Quin[8] concurred from his experience that sporadic cholera convulsions occur in peracute cases.

The development of convulsions in pigs affected with hog cholera is not a recent observation. Brunschwiler[9] observed that an animal may run in circles first to the right and then to the left, subsequently throwing itself down and going into a "cramp." It would draw itself backward with an opening and closing of the mouth. Dilated pupils and pushing movements with the front legs were also observed.

Seifried[10] reported the experimental production of 39 cases of encephalitis in swine characterized by histological changes in the brain. Nervous symptoms of varying degrees were seen. They neither made it clear, however, whether these nervous symptoms were severe enough to have been called convulsions, nor whether they had maintained such activity through serial animal passages.

Hutyra *et al.*,[11] in discussing hog cholera, stated that "Exceptionally there occur convulsions and various compulsive movements with marked lethargy. Attacks of delirium also occurred concurrent with hemorrhages between the meninges or into the brain substance. (Usually, however, histologically demonstrable encephalomyelitis is symptomless)."

Encephalitic symptoms were reported with other diseases of swine involving a number of animals in a drove. Ray[12] observed exaggerated nervous symptoms in swine infected with streptococci. Hofferd[13] stated that, although convulsions may be exhibited in cholera infections, they also are seen in a number of other diseases, including dietary deficiencies. It is possible that the author was referring here to calcium-deficiency tetany.

Kaplan[14] described encephalitic clinical reactions of swine infected with "porcine encephalomyelitis" or Teschen's disease. Such animals showed incubation periods of three to thirty-one days, nervous excitement, and paralysis. The animals would fall on their sides, squeal, and go into convulsions. Upon recovering, they would again be able to stand. The paralytic stage came gradually. The animals lost control of their hind legs and exhibited a "dog sitting" posture. Approximately, 50 per cent of affected animals died during the first two weeks. Early recoveries in nonparalyzed animals occurred in one to two weeks. Some animals remained paralyzed for weeks.

Reichel[15] compared the increase of virulence of hog cholera virus in serial inoculation to fixed rabies virus which was subjected to serial animal passage until a maximum virulence was obtained. Wright and Habel[16] demonstrated that substrains of Pasteur-fixed rabies virus were neither identical in their antigenicity, in their ability to overcome rabies immunity in immunized mice, nor in the incubation periods, duration of symptoms, or lesions of the type of disease they produced.

MATERIALS AND METHODS

The virus used in this experiment was isolated from a pig following field-vaccination failure. There was no history of convulsions, but the death loss was high. One passage of the virus was made prior to the serial intracranial inoculation of 6 pigs. In this passage, 1 animal which had been inoculated intradermally showed convulsions on the eleventh day after receiving the virus.

The brains from infected animals were held at —20 C. until the day before inoculations were to be made. Where possible, the brain selected for the experimental serial passage was one that had been removed from the first animal to die in the last-completed passage of the series. The first animal to die was usually one of those which had suffered convulsions. Thus, selection was made on the basis of early death and convulsions.

The brain was removed from the deep freeze and allowed to thaw at 42 C. A portion, never more than half of the brain, was accurately weighed and ground in a mortar with sterile quartz sand. Brain-heart infusion broth was added in sufficient quantity to make a 20 per cent suspension of the tissue. This suspension was centrifuged at 3,000 to 4,000 revolutions per minute for twenty to thirty minutes in an International refrigerated centrifuge and filtered through a Seitz E-K filter pad. The higher speed and longer period facilitated filtration. Bacteriological tests of the finished product were made by inoculating artificial mediums, animals, and chicken embryos.

Intracranial inoculations were made with the animal under anesthesia. Surital sodium (Parke Davis & Co., Detroit, Mich.) in a 1 : 25 aqueous solution was injected either intravenously, via the anterior vena cava, or intraperitoneally.

The hair on the pig's forehead was clipped closely, and the area was scrubbed with alcohol and painted with iodine. An incision was made on the medial line slightly above the point where an imaginary line drawn from the right eye to the base of the left ear crossed a similar line drawn from the left eye to the base of the right ear. The skin of the forehead was then pulled to one side, bringing the incision approximately ½ in. lateral to the medial line. At this point, the skull was trephined, a ½-in. needle was inserted into the cerebrum, and 0.5 cc. of the virus injected.

Subcutaneously inoculated swine had 1.0 cc. of virus injected into the left axillary space. Serum, when given, was injected into the right axillary space.

Observations were made each morning. Temperatures were taken, actions observed, and blood for cell counts obtained by bleeding either from the ear or the tail. The total white cell counts (t.w.c.c.) were made later in the laboratory. Necropsy was performed

upon all dead or moribund individuals in the experiment. Organs from which tissues were taken for sectioning included the cerebrum, cerebellum, and brain stem, as well as many other tissues. A bacteriological check on all swine at necropsy was made by culturing the heart blood, the brain, and the large intestine.

RESULTS

Encephalitic Symptoms. — The primary characteristic of the disease, caused by this virus, was an increased manifestation of nervous symptoms between the third and

Fig. 1—An early stage of convulsion. Hind legs are stiffened but forelegs are drawn tightly to the thorax. Note the tightly closed eyes.

eleventh day following the inoculation of the virus. This symptom was exhibited by pigs inoculated with either the filtrate of brain suspension or blood, and by either intracerebral or subcutaneous routes. Usually, this symptom was noticed first on the fifth or sixth day, starting as convulsive seizures of one-minute to two-hour intervals. In some of the more severely affected animals, the intervals became shorter and shorter until almost continuous convulsive activity was evident. Usually at this time, the animal was quite exhausted and its activity was more feeble. The convulsions commonly followed a pattern. The animal stiffened, the head was thrown back, the eyes closed tightly, and the legs extended rigidly. The subject remained in this position, exhibiting a muscular tremor for ten to fifteen seconds (fig. 1). This was followed immediately by an easing of muscular tension, permitting extended running action while the animal remained on its side (fig. 2). Loud squealing sometimes accompanied the running movements which lasted for approximately thirty to forty seconds. With the cessation of the convulsive action, the animal relaxed slowly but breathed heavily and displayed a wild look in its eyes (fig. 2). If quite exhausted upon cessation of convulsions, the animal would frequently lie on its sternum with a depressed attitude.

Upon becoming prostrate, the pig would lie on one side only. Any attempt to place it on the opposite side was met with violent efforts to regain the original position. The animal would struggle in a furious manner. Usually, the original position would be attained unless the animal was too weak to turn itself. The animal would then lie quietly until the next convulsion.

Early death did not follow the seizures. Many times, there was complete recovery from nervous symptoms. The animal even recovered so far as to be able to get up on its feet again, to take nourishment, and to live for several days. One animal survived for more than a month following convulsive seizures.

More commonly, death occurred within two or three days after the onset of nervous symptoms. The animal did not always continue to show these symptoms until the time of death.

Tables 1 and 2 show that the occurrence of convulsions was not related to the route of inoculation. Of 52 intracranially inoculated animals, 22 (42.3%) displayed severe nervous symptoms, whereas 11 of 21 subcutaneous inoculated animals (52.1%) reacted in a similar manner. There seemed to be a somewhat cyclical variation in the incidence of convulsive activity through-

Fig. 2—Late stages of a convulsion. Running movements are beginning and the animal has a "wild look" in its eye.

out the series of passages. In some passages all pigs were affected, but in other passages none was affected.

A small number (less than 10%) of the animals listed in table 1 as having nervous symptoms did not exhibit actual convulsions. These showed chorea-like movements of the head or legs, or winced spasmodically as if in severe cerebral pain. Since, in a few cases, the cessation of convulsive activity had been observed to occur within eight to ten hours from its onset, it may be that these few cases were passing through the end of a convulsive period. Shivering and nervous movements were common in most dying animals but were not included in the table. Observations usually were not made more than twice a day, due to the distance between the laboratory and the quarantine quarters. It may be that with closer observation the number of convulsion cases reported would have been greater.

TABLE 1—The Relation of Nervous Reactions to the Course of the Disease in Serial Intracranial Swine Passages

Passage (No.)	No. with severe nervous symptoms over No. injected	Day nervous symptoms observed	Day of first death	Average days of survival
1	2/3	6, 8	8	15.7
2	0/2	.....	4	9.5
3	2/3	6, 7	6	25.0*
4	3/3	4, 5, 6	6	7.7
5	0/1	.....	8	8.0
6	2/2	6, 8	6	15.5
7	0/3	.....	21	26.7
8	2/2	9, 10	10	12.0
9	3/3	3, 5, 6	4	8.3
10	0/2	.....	9	10.0
11	0/4	.....	8	9.0
12	2/6	3, 5	4	11.3
13	2/6	8, 11	9	12.2
14	0/6	.....	10	12.0
15	3/4	7, 7, 9	7	10.5
16	1/2	4	5	7.0

*One animal showing no convulsions survived forty-eight days.

In 16 serial intracranial swine passages of the virus, there was a tendency toward decreased encephalitic symptoms in passages ten through 14, as shown in table 1. However, pigs in passages 15 and 16 again showed marked convulsive activity. The over-all occurrence of nervous symptoms dropped from 63.6 per cent at passage nine to 42.3 per cent of all animals inoculated intracerebrally up to and including passage 15.

It is interesting to note in table 1 that convulsions were associated with the acute course of the disease. The average day for the first death to occur in groups showing convulsions was 6.3 days following inoculation. The average day of the first death in groups not exhibiting convulsions occurred ten days postinoculation. Thus, there appeared to be a correlation between the acute course of the disease and the occurrence of convulsions.

Convulsions occurred from the third to the thirteenth day after inoculation (tables 1 and 2) and averaged 6.7 days in duration. It was observed that convulsions in 80 per cent of the pigs showing such reactions occurred between the fifth and the ninth day after injection of the virus. Death of pigs suffering convulsions occurred on the average of 9.6 days following inoculation.

Goose-stepping was noticed in several animals. Possibility of pantothenic acid deficiency existed only if the prolonged period of anorexia was sufficient to deplete any reserve the animal might have had. The diet provided adequate B vitamin supplementation for healthy pigs.

Perversion of appetites, in the form of eating straw in preference to palatable feed, was also observed. Animals in a weakened condition would sometimes gnaw on the troughs, the walls, and even the attendant's boots. Yet, if placed where good feed or water were available, the pigs would often ignore both. At such times, the pigs appeared to be almost blind and walked with stumbling movements.

Diarrhea, following constipation, was common in many animals from the eighth day until death. The feces were usually yellow in color but frequently were black from partially digested blood. In a few cases, unaltered blood was defecated.

TABLE 2—The Relation of Nervous Reactions to the Course of the Disease in Subcutaneous Inoculated Swine

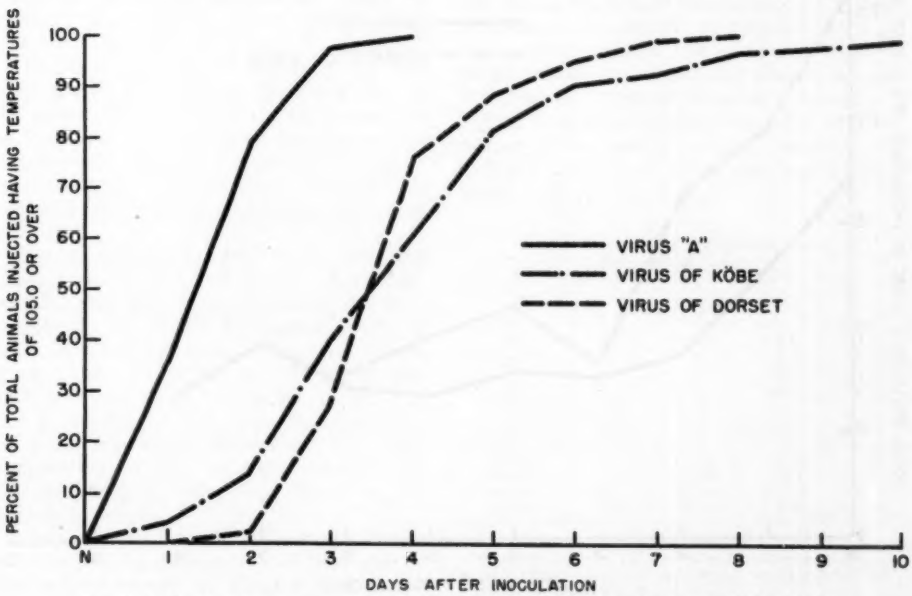
Virus of intracranial passage (No.)	No. with severe nervous symptoms over No. injected	Day nervous symptoms observed	Day of first death	Average days of survival
3	3/3	6, 8, 8	9	10.3
4	1/3	5	....	10.7
5	0/1	....	14	14.0
6	1/2	8	6	7.0
8	2/2	8, 9	8	9.0
9	2/3	5, 6	7	8.7
10	2/7	7, 13	13	13.6

During the colder months, when heat was provided in the pens, the mucous exudate dried on the nostrils of pigs which were sick seven days or longer. The exudate plugged the nostrils so that frequently the animals had great difficulty in breathing. This did not occur so frequently in later experiments.

Temperature Reactions.—The early body temperature rise in swine following inoculation with virus "A" denoted a short incubation period and suggested high virulence. In later passages, the temperature reactions on the first and second days appeared more frequently than in earlier passages. Graph 1 shows the temperature reactions caused by virus A as compared to reactions observed by Dorset[17] and Köbe[2]. Only those animals upon which daily temperatures were taken were included in the virus A curve. Graph 1 demonstrates that, on the first day following inoculation with virus A, 35.5 per cent of 39 animals showed a temperature of 105.0 F. or higher. Reactions in 79.5 and 97.4 per cent of the animals appeared the second and third days, respectively, when inoculated with virus A. This is compared to Köbe's findings of 4.5, 14.0, and 40.5 per cent temperature reactions on the first, second, and third days, respectively, after inoculation. Dorset's observations of 0, 2.25, and 28.0 per cent temperature reactions on the first three days following inoculation are also compared. Commercial hog cholera virus obtained locally and inoculated into 1 pig caused a rise of temperature over 105.0 F. on the fourth day.

Effect of Virus on Total White Cell Count.—The total white cell counts also indicated a short incubation period. A curve constructed from the median of daily t.w.c.c. on 53 animals (graph 2) shows a rapid drop on the first and second days following inoculation with virus A. This curve is compared on the same chart with curves based on the daily t.w.c.c. of animals injected with commercial virus II. The curve of the virus A animals shows evidence of a much more acute reaction than that of the animals infected with commercial virus. In 15 serial intracranial passages, there was no indication of a decrease in the leukopenic response to virus A. Commercial virus II is represented by the average t.w.c.c. of 6 animals. It was noted that, throughout the experiments, the short incubation period of virus A was reflected in the rapid drop of the t.w.c.c.

In various experimental phases reported



Graph 1—Temperature reactions produced by viruses of Köbe and Dorset and Virus "A".

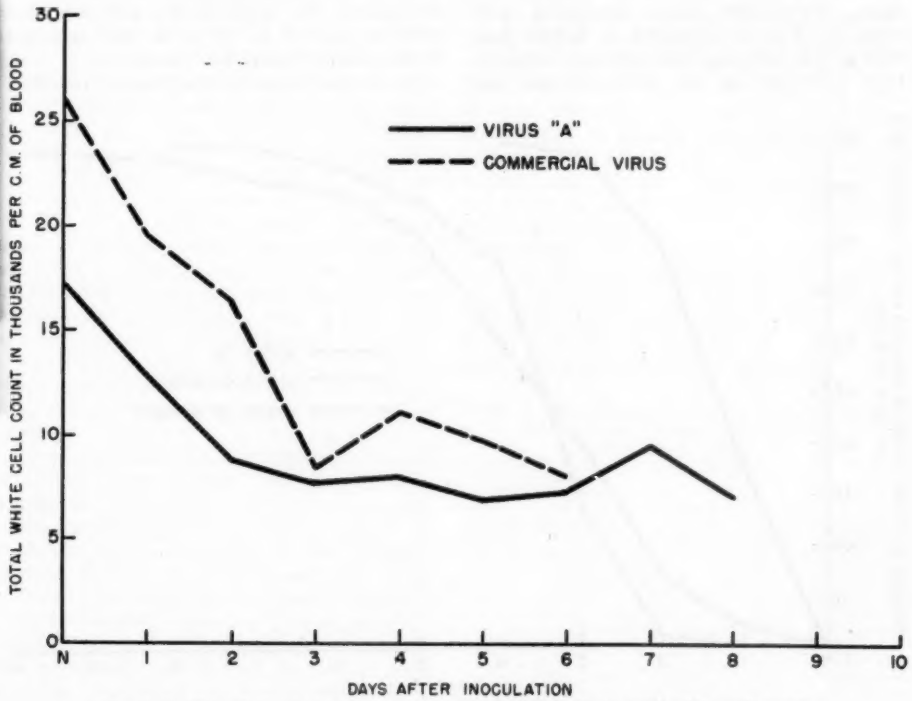
in this paper, the t.w.c.c. was used as a means of following the course of the virus infection. In conjunction with the use of body temperatures, the t.w.c.c. is an effective laboratory aid for studying the hog cholera virus.

Immunity Reactions.—The effectiveness of Bureau of Animal Industry (BAI) experimental serum No. I and of a commercial serum was tested in a series of immunity experiments. Table 3 summarizes the results of experiments with the BAI serum and demonstrates its relative ineffectiveness in neutralizing virus A. In lightweight pigs (35-40 lb.), all animals became sick when injected subcutaneously with 2.0 cc. of the virus and intramuscularly with 15 cc. of the BAI serum. Four out of 7 (57.1%) of these died. One of 3, 35- to 40-lb. animals sickened but did not die when 30 cc. of serum was used to neutralize the virus.

The commercial serum was much more effective but not completely so when 15-cc. amounts were injected into 60-lb. swine, as shown in table 4. Although this dose of serum is small, it is the BAI[18] regulation test dose for this size pig in commercial serum production.

Two routes of virus inoculation, intracerebral and subcutaneous, were used in the immunity experiments. Of the two, the intracerebral route required the least serum for neutralization. This was at least partially due to the smaller intracerebral virus dose, which was 0.5 to 1.0 cc., while the subcutaneous virus injections were made in 2.0-cc. amounts.

Exposure of Vaccinated and Recovered Pigs.—Four vaccinated pigs were exposed to virus A. Pig 4 had been vaccinated approximately two months prior to the experiment with commercial serum and virus and was in good health. As a contact control, the pig had a normal t.w.c.c. of 19,550. On the eighth day following the animal's contact exposure with the original field case virus A, the t.w.c.c. dropped to 12,250 and the temperature rose from 102.4 F. Both t.w.c.c. and temperature returned to



Graph 2—The effect of virus "A" and commercial virus II on total white cell count.

a more normal range for three days. Then, the t.w.c.c. dropped again to 11,600 on the twelfth day, 8,500 on the thirteenth day, and rose to 9,600 on the fourteenth day. The temperature again rose to 104.0 F.

TABLE 3—Immunity Experiments Using Bureau of of Animal Industry Experimental Serum

Weight of pigs	Routes of inoculation of virus	Amount of serum	No. sick over No. injected	No. died over No. injected
35-40 lb.	IC*	10 cc.	3/3	1/3
35-40 lb.	S**	15 cc.	7/7	4/7
35-40 lb.	S	30 cc.	1/3	0/3

*Intracranial. **Subcutaneous.

on the fourteenth day. Thereafter, the t.w.c.c. and temperature returned to normal, but the animal developed a yellowish diarrhea, lost its appetite, had a severe exudative conjunctivitis, and lost much weight. Recovery was uneventful after approximately ten days of illness.

Three additional pigs, vaccinated with commercial serum and virus approximately two months prior to this experiment, were injected subcutaneously with 0.5 cc. of virus A (20% suspension of brain in broth). The reaction was a marked drop in t.w.c.c. in 2 of the 3 pigs, from 24,100 to 10,400 on the fourth day in 1 pig and from 20,350 to 8,950 on the third day in the other. Both pigs developed a diarrhea on the eighth day following inoculation but subsequently enjoyed an uneventful recovery. All 3 pigs showed body temperatures of 104.4 to 104.8 F. following the virus injection.

Pig 4, completely recovered from the exposure to virus A two months previously, was injected with 3 cc. of the 20 per cent suspension of virus A-infected brain in broth. A slight drop in t.w.c.c. was noted, from 19,850 to 16,400 on the third day with a return to 18,750 on the fourth day and 20,000 on the fifth day. No significant variation was noted thereafter. The body temperature of this animal, however, did rise to 104.0 F. following the virus injection.

Gross and Microscopic Pathology.—It can be stated quite definitely that the type of hog cholera caused by virus A was essentially a disease of endothelial tissues, characterized by hydropic degeneration, endothelial proliferation, perivascular cuffing and, in many areas, hemorrhage. With few exceptions, the lesions and symptoms observed in hog cholera infections apparently resulted from degenerating or proliferating endothelial tissues.

Virus A infection was characterized by the severity of lesions produced in the animals, particularly the severe hemorrhages of the kidneys and lymph nodes, numerous infarcts of the spleen, extensive ulceration of the stomach and large intestine, and pronounced congestion of the brain.

Sections of the brain, including the medulla, thalamus, and cerebrum, were examined microscopically for lesions. The most significant changes were congestion, perivascular cuffing, and endothelial proliferation with partial occlusion of the vessels. Foci of microglia were observed in scattered areas in a few sections. The most pronounced reactions occurred in the medulla and thalamus, with the latter showing the highest incidence of lesions. It appeared that the severity of lesions was greatest in sections taken between the tenth and fourteenth day after inoculation.

The stomach contained little ingesta at the time of necropsy. In 8.3 per cent of the cases, petechial hemorrhages in the mucosa were observed. In 3 cases (3.6%), there were petechial "paint brush" hemorrhages of the gastric serosa. Gastric edema occurred only twice in 84 cases (table 5).

Mild to moderate, acute catarrhal inflammation of the small intestine occurred in 15.5 per cent of the cases, but was a relatively unimportant lesion.

TABLE 4—Immunity Experiments Using Commercial Serum

Weight of pigs	Routes of inoculation of virus	Amount of serum	No. sick over No. injected	No. died over No. injected
80 lb.	IC*	5 cc.	2/2	2/2
60 lb.	S**	15 cc.	2/2	1/2
60 lb.	IC	15 cc.	1/2	0/2
35 lb.	S	15 cc.	3/4	0/4

*Intracranial. **Subcutaneous.
Virus dose was 2.0 cc. in subcutaneously inoculated animals and 0.5 to 1.0 cc. in intracranially inoculated animals.

Acute, catarrhal colitis was observed in 21.4 per cent of the cases, and diffuse necrotic colitis and cecitis were seen in 16.8 per cent. All necrotic enteritis cases were acute to subacute. Button ulceration

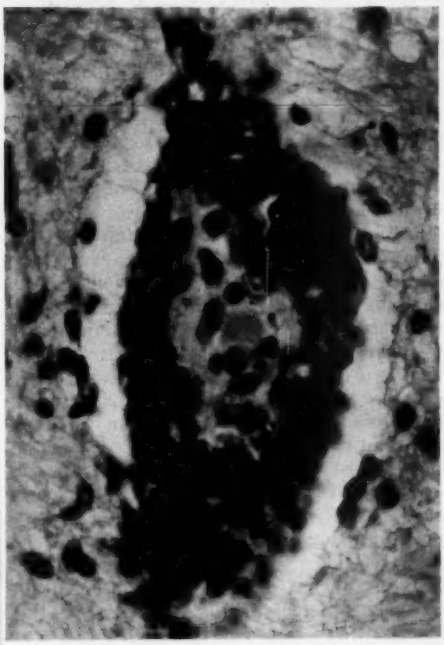


Fig. 3—Arteriole in the medulla oblongata showing heavy perivascular cuffing with partial occlusion of the vessel lumen. Virus "A"—infected. Hematoxylin-eosin stain. x 800.

of the colon was evident in 20.2 per cent of the cases. Ulcers were generally in an early stage of development. Nutritional enteritis-type lesions were observed in 3 cases. The lesions were characterized by fecal plaques adhered to the intestinal mucosa by mucous strands, as described by Dunne, *et al.*[19] Only 10.7 per cent of the animals showed colonic petechiation. In an additional three instances, there was hemorrhage into the intestinal lumen.

Since the lesions of most of the organs conformed to textbook descriptions of the disease, little will be said about them other than to stress the severity of lesions observed in many cases.

The lymph nodes were congested or showed evidence of varying degrees of hemorrhage in more than 96 per cent of the cases studied. Hemorrhage was generally moderate to severe and more commonly diffuse than peripheral. Both types of hemorrhage were frequently observed in the same animal. In such cases, the cervical, pharyngeal, and submaxillary lymph nodes displayed diffuse hemorrhage, whereas the mesenteric and associated lymph nodes more frequently showed peripheral hemorrhage. It was interesting to note that the diffuse type of hemorrhage was recorded in 71.4 per cent of the cases, whereas peripheral hemorrhage was observed in only 47.6 per cent of the cases.

Skin erythema was observed in less than 30 per cent of the animals. In this respect, however, consideration was given to the fact that the majority of the animals inoculated had black skins.

Tonsillitis occurred in a surprisingly high number (38.1%) of cases. This may have been partially due to the use of wheat straw bedding for a short time when other bedding was not available. The barbed wheat beards induced pronounced abscess formation in the tonsillar tissue.

Myocardial degeneration appeared in

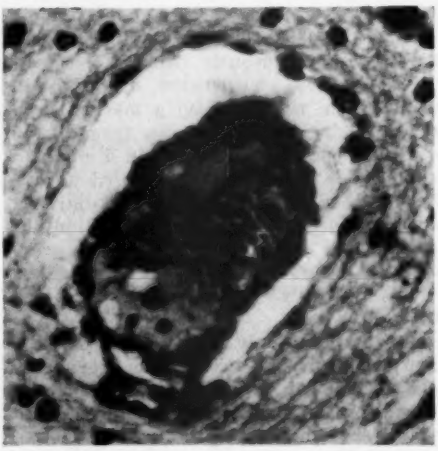


Fig. 4—Blood vessel from medula oblongata of an animal infected with a commercial virus. Little or no perivascular cuffing was evident in this section. Hematoxylin-eosin stain x 730.

35.7 per cent of the cases upon gross examination. Microscopically, coagulation necrosis was observed in myocardial fibers, particularly in those of the auricles.

Bronchopneumonia was observed in 58.3

per cent of the animals examined at necropsy. Approximately 60 per cent of this number were mild in character. Only 7 of the 49 pneumonia cases were of a severe nature. In many instances, the lung was unilaterally affected, indicating a hypostatic type of pneumonia. In one instance, a very severe acute type of local necrosis was observed. Areas of the lung 2 to 4 cm. in diameter were completely lysed, as if the area had been necrotic and sloughed away. *Pseudomonas aeruginosa* was isolated from the heart blood of this animal and may have played an important part in the development of the lesions. Pulmonary ecchymosis was observed in 15.7 per cent of the necropsies, and more than half of these were severe in nature.

Lesions of the kidney provided some of the more spectacular changes observed in animals infected with virus A. Hemorrhages of the cortex varied from mild petechiation to severe ecchymosis. In some instances, mild petechiation of the cortex was associated with severe diffuse hemorrhage of the pyramids. In other cases, the cortex would show severe petechiation with less severe hemorrhage of the pyramids. The most pronounced lesions were found in those cases in which severe ecchymotic hemorrhages appeared in the cortex of the kidney and diffuse hemorrhage was present in the pyramids. Hemorrhage of the pyramids was observed in 28.6 per cent of the postmortem examinations.

Although a thorough microscopic examination of kidney sections was not undertaken, the few sections examined revealed extensive hemorrhage into the interstitial tissue. The cells of the tubules were undergoing coagulation necrosis with some karyolysis already evident.

Splenic infarction was quite common and rather severe in nature. Multiple infarctions were the rule, occurring both on the borders and in the parenchyma.

Purity Tests.—Bacteriological examinations revealed no *Salmonella choleraesuis* in the heart blood, brain, or intestinal tissues. No streptococci were isolated from brains of the animals, even though particular attention was given to those animals which had shown convulsions.

Subcutaneous inoculations of infected swine brain suspension into rabbits failed to effect any reaction, thus eliminating the possibility of the presence of Aujeszky's disease virus. The symptoms, course of the disease, mortality, and histological examination of the brain excluded the possibility of the presence of Teschen's disease virus. Chicken embryo inoculations were negative.

DISCUSSION

The two main objectives in this investigation were to study the characteristics of a variant strain of virus solated from pigs dying as the result of a field-vaccination failure and to maintain these characteristics over a number of serial passages.

The outstanding characteristics of this virus, referred to as virus A, have been its ability to produce a disease with encephalitic symptoms, short incubation period, a short course, and severe lesions. It does not necessarily means that encephalitic symptoms manifested by convulsive seizures identify this virus as one of the variant

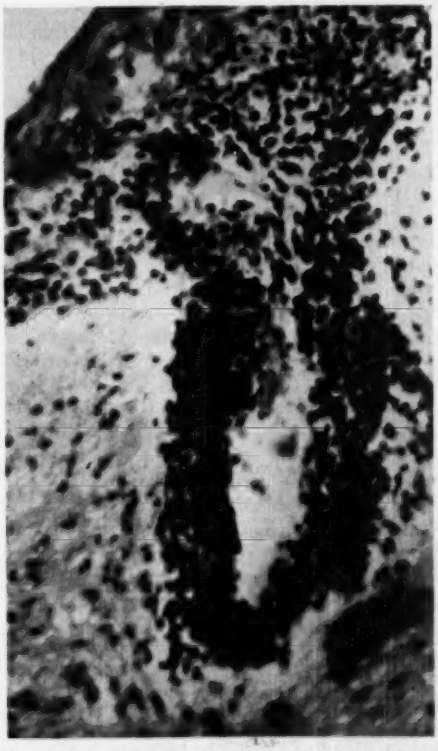


Fig. 5—A peripheral vessel of the medulla oblongata showing perivascular cuffing, but also apparently distended. Virus "A"—infected. Hematoxylin-eosin stain. x 300.

types observed during the 1949-50 period of increased vaccination failures. The convulsions observed in virus A-infected animals were very similar to those described by Brunschwiler.[9] Convulsions occurred in peracute cases of virus A-infected animals much in the manner mentioned by Quin.[8] Hutyra, *et al.*[11] acknowledged that in hog cholera cases "exceptionally there occur convulsions and various compulsive movements with marked lethargy." This helps to confirm the association of convulsions with hog cholera infections but further accentuates the fact that frequent observation of such phenomenon, as in virus A-infected animals, is unusual. Hofferd's[13] suggestion of the relationship of dietary deficiency to convulsive seizure was interpreted to mean possibly calcium deficiency. The injection of 25 cc. of calcium gluconate intravenously between virus A-induced convulsive seizures did not prevent the recurrence of convulsions several minutes later. Ray[12] discussed the encephalitic reaction of streptococcic-infected swine, but cultures of the brains of virus A-infected animals revealed no streptococci or other pathogens.

Subcutaneous injections of rabbits with brain suspension of infected swine produced no reaction and eliminated the possibility of Aujeszky's disease. The course and mortality of the infection, as well as the examination of histological sections of the brain and cord, eliminated Teschen's disease.

TABLE 5—Summary of the Gross Lesions of 84 Experimentally Infected Hog Cholera Pigs

Lesion	Mild	Moderate	Severe	Total	% of total
Conjunctivitis	10	24	25	59	70.2
Erythema	11	9	5	25	29.8
Subcutaneous hemorrhage	..	1	1	2	2.4
Lymphatic peripheral hemorrhage	17	15	8	40	47.6
Lymphatic diffuse hemorrhage	12	33	15	60	71.4
Tonsillitis	11	13	8	32	38.1
Epiglottis—petechiation	13	5	2	20	23.8
Hydrothorax	5	9	0	14	16.8
Hemothorax	2	3	0	5	6.0
Hydroperitoneum	2	6	1	9	10.7
Thymus—petechiation	..	..	1	1	1.2
Hydropericardium	6	3	1	10	11.9
Epicardial petechiation	2	5	4	11	13.1
Myocardial degeneration	19	9	2	30	35.7
Coronary occlusion	1	2	0	3	3.6
Fibrinous pericarditis	0	3	1	4	4.8
Pleuritis	0	2	0	2	2.8
Bronchopneumonia	30	12	7	49	58.3
Pneumonic ecchymosis	1	5	7	13	15.7
Interstitial edema	0	0	1	1	1.9
Atelectasis	3	2	0	5	6.0
Peritonitis	1	2	0	3	3.6
Gastritis	16	13	9	38	45.2
Gastric edema	1	1	0	2	2.4
Gastric serosa petechiation	0	2	1	3	3.6
Gastric mucosa petechiation	1	4	2	7	8.3
Enteritis—small intestine	9	3	1	13	15.5
Severe intestinal serosal and mucosal petechiation	0	1	0	1	1.2
Colitis, cecitis, diffuse necrotic	6	5	3	14	16.8
Acute colic ulceration	0	0	1	1	1.2
Button ulcers	7	5	5	17	20.2
Colonic petechiation	1	4	4	9	10.7
Nutritional enteritis	3	0	0	3	3.6
Acute catarrhal colitis	9	7	1	18	21.4
Hemorrhagic colitis	1	..	2	3	3.6
Splenic infarction	5	13	5	23	27.4
Renal cloudy swelling	9	3	2	14	16.8
Renal petechiation cortex	25	19	10	54	64.3
Renal cortex ecchymosis	3	0	10	13	15.5
Renal pyramids ecchymosis	8	7	9	24	28.6
Nephritis	0	2	0	2	2.4
Hepatic fatty metamorphosis	0	6	0	6	7.1
Cholecystic congestion	2	1	0	3	3.6
Cholecystic petechiation	3	3	3	9	10.7
Cholecystic ecchymosis	2	2	1	5	6.0
Hepatic scars	2	14	4	20	23.8
Ascariasis	2	6	3	11	13.1
Cystic petechiation	40	22	4	66	78.6
Cerebral congestion	15	31	15	61	72.6
Petechiation of omentum and mesentery	0	0	1	1	1.2

The short incubation of virus A infections was shown by the rapid body temperature rise and the quick drop of t.w.c.c. A comparison of the rapid rise of temperature in virus A-infected pigs with results obtained from the use of other viruses by other investigators showed that virus A had a much shorter incubation period than viruses studied by Dorset,[17] Lewis and Shope,[20] and Köbe.[2]

The course of the disease was marked by frequent acute deaths. Deaths as early as three days and less rarely at five and six days occurred. However, some animals survived as long as forty-eight days. The mortality was 100 per cent, for all animals either died or became unable to rise and take nourishment and were destroyed. There were no recoveries in animals not receiving serum.

Dale *et al.*[6] tested a variant strain of virus with simultaneous injection of BAI experimental serum No. I in swine and found the serum to be ineffective in 15- to 20-cc. doses. This serum was also ineffective when used simultaneously with virus A in similar doses. While the percentage of protection afforded by the serum against virus A appeared greater than against the variant strain of Dale, it will be observed that lighter pigs (35 to 40 lb.) were used in the present experiment than in the experiment of Dale *et al.*, in which pigs from 40 through 150 lb. were used.

Difference in types of inoculating materials must be considered in all comparative tests. Without exception, the viruses with which virus A has been compared have been of blood origin. With few exceptions, virus A has been of brain tissue origin. However, it was used throughout the experiment in a 20 per cent suspension in broth or saline. Theoretically, this would appear to make it more dilute than virus of blood origin, since the virus seems to have an affinity for vascular endothelial tissue.

The fundamental reason for lack of protection afforded by a serum of known potency is not clear. Two factors that may be responsible are differences either in antigenicity or virulence of the virus. In view of the lack of sufficient evidence indicating antigenic variation of virus A, it must be recognized that all the immunological differences observed could be explained on the basis of increased virulence. This was emphasized by Ruppert,[22] who suggested that great variations exist in the virulence of hog cholera virus. He believed that an immunity brought about by a weak virus could be overcome by a strong virus.

A study of the pathological changes observed in animals infected with virus A verified the findings of Seifried and Cain[23] and Bueno,[24] both of whom were convinced that swine fever was a disease of the reticuloendothelial system. Lesions of the brain have further demonstrated the effects of virus A on vascular endothelial cells. The hydropic degeneration and proliferation of vascular endothelial cells with perivascular cuffing conformed to the findings of Helmbolt and Jungherr.[25] These lesions contributed markedly to the constriction and occlusion of the lumen of involved capillaries and arterioles. The constriction of the vessel lumen would slow the blood stream and contribute to the sludging action described by Beamer *et al.*[26] This partial occlusion of blood vessels may explain the convulsive activity exhibited by the swine in this experiment.

The somewhat frequent, severe medullary hemorrhages seen in the kidneys of virus A-infected animals exemplified, in a way, the severity of lesions produced by this virus. Dorset[27] described ecchymotic hemorrhages and clots in the pelvis as being seldom seen in chronic hog cholera but common in acute cases. Seifried and Cain,[23] however, rarely found petechial and ecchymotic hemorrhages in the medulla and pelvis.

We can not be certain that the variation observed in the hog cholera virus maintained in this laboratory is antigenically the same as that of the viruses tested by the BAI, although we feel that the immunological response to virus A closely follows the findings of Dale *et al.*[6] Neither can we be certain, at this point, that the virus is antigenically different from the strains of virus widely studied in Europe and in the United States.

Virus A reproduced in the experimentation described in this paper, however, has maintained characteristics which, at this time, must be considered as being those of a variant in either virulence or antigenicity. Serial passage of virus A has been maintained throughout 16 intracranial swine passages without losing these characteristics.

SUMMARY AND CONCLUSIONS

1) An encephalitic strain of hog cholera virus was isolated and maintained through 16 intracranial passages in pigs without the loss of ability to produce encaphalitic symptoms or other characteristics.

2) Encephalitic symptoms occurred just as frequently in subcutaneously inoculated swine as in intracranially inoculated ones.

3) This virus, designated as virus "A", produced a short incubation period, as indicated by the increased body temperature of inoculated pigs to 105.0 F. or higher twenty-four to seventy-two hours following inoculation of the virus.

4) A short incubation period was further indicated by the rapid drop of total white cell counts twenty-four to seventy-two hours following inoculation.

5) The course of disease caused by virus A was short, with deaths occurring as early as three to five days following inoculations.

6) In 100 animals inoculated, there were no recoveries. All animals died or were moribund when destroyed.

7) Incomplete protection against virus A was provided by Bureau of Animal Industry experimental serum No. I.

8) Commercial serum did not give complete protection in regulation serum test doses.

9) Thalamic lesions were evident in 100 per cent of all cases studied. Severity of lesions appeared greatest in animals dying between the tenth and the fourteenth days.

10) A commercial virus tested in pigs produced almost no lesions of the brain.

11) Hydropic degeneration and proliferation of the vascular endothelium appear to be the primary pathological changes, with hemorrhages and other lesions apparently secondary or the result of these changes.

12) No pathogenic bacteria other than *Pseudomonas aeruginosa* were isolated from the brain, blood, or intestine. This organism was isolated from 5 cases.

ADDENDUM

The recent work of Gochenour *et al.* with *Leptospira pomona* infections of swine as reported in the April, 1952, Journal of Veterinary Research suggested the possibility of the presence of that organism in the material or animals used in the serial passage of the strain of hog cholera virus discussed in this paper. However, treatment of each 100 cc. of passage material with 200,000 units of crystalline penicillin G potassium and 17.5 mg. of dihydrostreptomycin sulfate failed to decrease the occurrence of convulsions. More specific evidence of the absence of the organism was found in the negative microscopic agglutination reactions to *L. pomona* of several serums from infected, immune, and normal animals used in the experiment.

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Eperythrozoonosis in Swine—Filtration Studies

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Literature

In previous studies,[1,2,3] it was reported that a hemolytic anemia of swine (synonyms : "ictero-anemia," "anaplasmosis-like disease") occurring in the Midwestern states was due to an acute infection with a blood parasite of the genus Eperythrozoon. The causative organism was designated *Eperythrozoon suis*. The disease was reproduced experimentally in splenectomized pigs. The course of parasitic infection and the resulting disease process in field and experimental cases was found to be identical to that of acute, experimental eperythrozoonosis in cattle and sheep. An additional parasite, designated *Eperythrozoon parvum*, was recovered from swine in experimental studies. This organism was found to be relatively nonpathogenic in experimental animals, and believed to be of no etiological significance in the ictero-anemia disease entity. Tyzzer,* Neitz,* and other authorities[3] concurred that these blood structures should be classified as Eperythrozoon.

Foote *et al.*[4] presented evidence indicating that an ictero-anemic condition in swine which they encountered was due to a filterable virus. The disease was termed "virus anemia," and the filterable agent called "the virus of Farley." It was reported that eperythrozoa were not associated with the disease in early experimental work. In later transmission studies, structures said to be similar to *E. suis* and *E. parvum* were observed following the inoculation of blood serum and urine filtrates into susceptible pigs. The authors concluded that the ictero-anemia disease entity was due to a filterable virus, and that eperythrozoa were either reactionary bodies or artifacts, or were coincidental with the disease—they had no pathognomonic significance.

The studies reported in this paper were undertaken in an attempt to recover the virus of Farley from experimental and field cases of eperythrozoonosis—a virus which, according to its discoverers,[4] possessed the following major properties: (1) nonpathogenic to unoperated, experimental swine and cattle; pathogenic in splenectomized pigs and calves; (2) readily transmitted by subcutaneous or intraperitoneal injections of blood serum from carrier pigs or of urine from acute cases; (3) readily passed through 12-lb. Mandler and possibly finer candles; (4) period of incubation from three to ten days; eperythrozoa may or may not be present; (5) cultivable in the yolk sac of chick embryos; (6) infectious agent does not survive freezing;[5,6] and (7) early cases beneficially treated with aricyl (Winthrop-Stearns, Inc.)

These studies confirm the observation of Foote *et al.*[4] that the usually nonpathogenic organism, *E. parvum*, is filterable; however certain qualifications must be made in statements that the pathogenic agent, *E. suis*, is filterable. It has been demonstrated that the virus of Farley is not present in uncomplicated cases of acute eperythrozoonosis.

Although the exact classification of Eperythrozoon is uncertain, no reliable evidence has been presented in the past to suggest that these structures are reactionary bodies or artifacts. In the most recent authoritative classification,[7] the genus Eperythrozoon has been placed with other closely related blood parasites in the group of organisms intermediate between the bacteria and viruses (order Rickettsiales, family Bartonellaceae).

Numerous organisms in the order Rickettsiales may be passed through the usual bacteriological filters, and although many have not been cultivated on cell-free mediums, it is generally agreed that these structures are probably viable microörganisms and not reactionary bodies or cell-reaction products. The rickettsial organisms are included in this order, and are divided into two groups according to their passage or retention by filters.[7]

Procedure

Techniques involved in filtration were given careful attention, particularly in the use of bacteria-tight filters, the amounts of negative pressure used, the duration of the filtration process, and the conditions under which the filtrates were held. Each filter was tested for bacteria retention immediately following the recovery of the serum filtrate. The test mediums consisted of 24-hour broth cultures of *Serratia marcescens* diluted in saline to McFarland nephelometer tube No. 1 and adjusted to a pH of 7.4. It

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was intended that this solution approximate, in both the number of viable organisms present and the pH of swine serum heavily infected with eperythrozoa. The number of eperythrozoa present in swine serums could not be determined; however in heavy infections, myriads of the parasites could be observed in smears made of flocculent material in the serum.

Many of the medium-porosity filters failed to retain completely *S. marcescens* under the conditions of testing. Serum filtrates were, therefore, held in some experiments (exper. 2, 3, 6, 7, 8, and 11; table 1) from fifteen to twenty hours at 5C. in order that preliminary sterility checks could be made on filtrates of the test medium. Adequate controls were included to insure that both *E. suis* and *E. parvum* remained infectious in serum or blood held under these conditions.

The test for the presence or absence of eperythrozoa in blood, serum, or serum filtrates consisted of the injection of the material into susceptible pigs followed by continuous microscopic blood examination and clinical observation of the inoculated animal.

MATERIALS AND METHODS

All pigs used in transmission studies were splenectomized. Blood film examinations were made on alternate days for three or more weeks following removal of the spleen to determine the presence or absence of latent Eperythrozoon infection. All animals were eventually proved susceptible to acute infection with *E. suis* by the injection of virulent material. Susceptible pigs were held in fly-proof stalls at a distance of about 50 yards from known carriers. Pigs harboring infections of *E. parvum* only were held in isolated fly-proof stalls.

Blood serum for filtration was obtained from clotted blood and poured into centrifuge tubes. The serum was then centrifuged twenty minutes at 2,500 to 3,000 revolutions per minute in an International centrifuge (size 1, SB).

Filtration was carried out with all of the materials at room temperature. The filtration equipment consisted of Mandler, Berkefeld, and Seitz filters with the usual accessories, flasks, and hose. A dial-type gauge, measuring vacuum in millimeters of mercury, was mounted on a 1-liter filtering flask and connected between the source of vacuum and the flask containing the bacteriological filter. All filtrations were carried out at negative pressures not exceeding 100 mm. of mercury. The duration of the filtration process ranged between ten and twenty minutes for each filtrate.

Mandler and Berkefeld candles were cleaned and flushed with distilled water only. Seitz discs were used without previous washing and were discarded following use. Filters were tested for bacteria retention as previously described, and cultures for sterility were made on tryptose blood-agar plates, dextrose broth, and anaërobic meat-mash mediums. Amounts of filtrate cultured consisted of 1 cc. on plates and 3 to 5 cc. in tubes. Observations of the tubes were continued for three weeks for possible growth.

All pigs receiving injections of blood, serum, urine, or chicken embryo material were inoculated both intraperitoneally and subcutaneously. The quantity of material was divided equally between the two sites. Temperatures, blood film examinations, and erythrocyte counts were made on alternate days during the first ten days following inoculations; thereafter blood film examinations only were made until the appearance of eperythrozoa or the conclusion of the experiment. Aseptic precautions were taken in securing blood and making injections in order to prevent accidental transmission. Hot air was used in the sterilizing of syringes and needles.

The initial carrier pigs used in these studies harbored strains of *E. suis* derived from 14 separate sources in Kansas and Iowa. Eight were from outbreaks of acute eperythrozoonosis and six were from apparently normal carrier swine. Susceptible pigs 31, 34, and 50 (table 1) were naturally infected carriers of *E. parvum*, in which the latent infection was discovered following splenectomy. Information concerning the source of *E. parvum* infection in other susceptible pigs is contained in the data.

In those instances in which transmission of *E. suis* occurred to pigs also susceptible to *E. parvum*, it could not be readily determined by microscopic examination of blood of the affected pig whether or not transmission of the latter parasite had also occurred. The prolonged prepatent period of *E. parvum*, plus the complete suppression of the parasite resulting from an interference phenomenon with *E. suis* tended to prevent its early observation in mixed infections. It was not possible to determine whether or not transmission of *E. parvum* occurred to animals already harboring a latent infection of the parasite, since splenectomized pigs are premune to the particular Eperythrozoon species harbored. *Eperythrozoon parvum* carriers, however, are fully susceptible to acute, clinical infection with *E. suis*.

The degree of Eperythrozoon infection varying from "rare" to "extremely numerous" is an arbitrary designation described previously.[1]

EXPERIMENTAL DATA

Transmission Studies with Blood, Serum, and Serum Filtrates.—The results obtained in the transmission of *E. suis* and *E. parvum* in blood serum filtrates are summarized in table 1. Splenectomized pigs were injected with the materials indicated in amounts varying from 4 to 20 cc. Information is given concerning the status (carrier or acute case) of the donor animal

or animals, and the microscopic blood findings in each at the time blood was obtained. In experiments involving more than 1 donor, serum was collected from blood pooled in approximately equal amounts. All carriers were known to harbor both *E. suis* and *E. parvum*. Only those results are included in table 1 in which the filters used completely retained *S. marcescens* upon filtration of the test bacterial suspension.

TABLE 1—The Infectivity of Blood Serum Filtrates from Carriers and Acute Cases of Eperythrozoon Suis and Eperythrozoon parvum

Source of blood or serum	Degree of Eperythrozoon infection	Filter	Pig injected	Results
Experiment 1				
Carrier	negative	8-lb. Mandler	35	Negative. Pig remained normal 30 days
Experiment 2				
Acute case	*E. suis* rare	Seitz EK	40	Negative. Pig remained normal 91 days
		9-lb. Mandler	39	Negative. Pig remained normal 82 days
Experiment 3				
		14-lb. Mandler	37	*E. parvum* recovered. Pig remained unaffected 152 days
2 carriers	negative			
1 carrier	*E. parvum* rare	Seitz EK	38	*E. parvum* recovered. Pig remained unaffected 150 days
Experiment 4				
3 carriers	negative	12-lb. Mandler double filtration		
2 carriers	*E. parvum* rare		41	*E. parvum* recovered. Pig remained unaffected 135 days
2 carriers	*E. suis* rare			
Experiment 5				
3 carriers	negative	N-Berkefeld	33	*E. parvum* recovered. Acute infection followed a 27-day incubation period
3 carriers	*E. parvum* rare			
Experiment 6				
Acute case	*E. parvum* scarce	Seitz EK	32	*E. parvum* recovered. Pig remained unaffected 85 days
Acute case	*E. suis* very numerous			
Acute case	*E. suis* frequent			
Experiment 7				
		Seitz EK	BW	Negative. Pig remained normal 142 days.
Acute case	*E. suis* numerous	N-Berkefeld	31*	*E. suis* recovered. Acute infection followed a 16-day incubation period
Experiment 8				
Carrier	negative	8-lb. Mandler	39	Negative. Pig remained normal 47 days.
		N-Berkefeld	40	Negative. Pig remained normal 56 days
		Seitz EK	43	Negative. Pig remained normal 128 days
		Serum @	36	Negative. Pig remained normal 49 days
		Whole blood @	34*	*E. suis* recovered. Acute infection followed a 10-day incubation period
Experiment 9				
		8-lb. Mandler	36	*E. parvum* recovered. Acute infection followed a 29-day incubation period.
		Two 8-lb. Mandlers	39	*E. parvum* recovered. Pig remained unaffected 97 days
3 carriers	negative	Seitz EK	40	*E. parvum* recovered. Pig remained unaffected 124 days
		N-Berkefeld	37*	Negative. Pig remained normal 112 days
		Whole blood @	32*	*E. suis* recovered. Acute infection followed a 13-day incubation period
Experiment 10				
		Seitz EK	BW	Negative. Pig remained normal 95 days
		N-Berkefeld	42	Negative. Pig remained normal 132 days
Carrier	negative	8-lb. Mandler	45	Negative. Pig remained normal 60 days
		Serum @	46	Negative. Pig remained normal 110 days
		Whole blood @	47	*E. parvum* recovered. Acute infection followed a 24-day incubation period
Experiment 11				
		Seitz EK	50*	Negative. Pig remained normal 78 days
		W-Berkefeld	44	*E. parvum* recovered. Pig remained unaffected for 99 days
2 acute cases	*E. suis* very numerous			
		14-lb. Mandler	41*	Negative. Pig remained normal 85 days
		8-lb. Mandler	48	*E. suis* recovered. Acute infection followed a 14-day incubation period
Experiment 12				
		Seitz EK	40*	Negative. Pig remained normal 75 days
2 carriers	*E. parvum* scarce	Two Seitz EK filters	42	Negative. Pig remained normal 66 days
1 carrier	*E. suis* rare	W-Berkefeld	BW	*E. parvum* recovered. Pig remained unaffected for 52 days
		14-lb. Mandler	39*	*E. suis* recovered. Acute infection followed an 11-day incubation period
		8-lb. Mandler	36*	*E. suis* recovered. Acute infection followed a 15-day incubation period
		Serum @	38*	*E. suis* recovered. Incubation period prolonged to 37 days by arsenical therapy
		Whole blood @	43	*E. suis* recovered. Acute infection followed a 7-day incubation period

*indicates that the pig was a carrier of *Eperythrozoon parvum* at the time the inoculation was made.
@ unfiltered material.

In the early studies, the results were entirely negative for the recovery of a pathogenic agent from filtered serums when bacteria-tight filters were used. Serums passed through leaky filter candles (6 instances), however, produced acute infections with *E. suis*. *Eperythrozoon parvum* only was recovered in a number of pigs following injection of filtrates from bacteria-tight candles. The organism appeared in rare numbers in the blood of inoculated pigs usually between the fourteenth and eighteenth days. The parasites increased in numbers to the twenty-first or twenty-sixth days and then gradually disappeared. Harmless recidivations occurred in some pigs.

As noted previously,[2, 3] *E. parvum* did not reproduce the acute ictero-anemia syndrome. Pigs which developed infection from filtrates remained unaffected for as long as 152 days until proved susceptible to acute infection with *E. suis*. Three exceptions occurred during the course of study. Acute eperythrozoonosis developed as a result of *E. parvum* infection in pigs 33, 36, and 47 following incubation periods of from twenty-four to twenty-nine days. This is in contrast to an average incubation period of 9.7 days in acute experimental infections of *E. suis*. That these acute cases were due to *E. parvum* was further shown by the inoculation of unfiltered blood and urine from the acutely affected cases into susceptible pigs or carriers of *E. parvum* only. Susceptible pigs receiving these injections developed harmless infections of *E. parvum*, while carriers of the parasite remained entirely normal.

As additional splenectomized pigs became available for use, a larger number were included in each experiment. Controls on the infectivity of unfiltered serum or whole blood were included to determine conclusively whether *E. suis* was removed by filtration or was not present in the serum. It was found that experimental transmission of *E. suis* could not be accomplished regularly with blood serum from microscopically negative carriers. On the other hand, *E. parvum* was usually present or appeared from time to time in small numbers in the blood of these carriers, and it was recovered with greater consistency from the serum.

Two failures occurred in the transmission of *E. suis* using unfiltered whole blood from known carriers. One of these occurred in experiment 10 (table 1), and the other is not included in the data. In both instances, *E. parvum* was recovered in an uncomplicated infection.

Eperythrozoon suis was passed through bacteria-tight filters in experiments 7, 11, and 12 (table 1). In experiment 7, passage occurred through the N-Berkefeld, but complete retention was obtained with the Seitz EK filter. In experiment 11, *E. suis* passed the 8-lb. but not the 14-lb. Mandler, the W-Berkefeld, or the Seitz EK filters. In experiment 12, passage was effected through the 8- and 14-lb. Mandlers, but not the W-Berkefeld or the Seitz EK filters. Cases resulting from transmission of *E. suis* in filtrates did not differ from those previously reproduced with whole blood or improperly filtered serum. The incubation period was somewhat prolonged, however, and varied from eleven to sixteen days. As in other acutely affected pigs, injections of neoarsphenamine produced specific and immediate control of the disease.

Eperythrozoon parvum was found capable of passing all of the filters of medium and fine porosity which were used. In experiments 4, 9, and 12, the parasite was passed on double filtration through the 12- and 8-lb. Mandlers, but did not pass in double filtration with the Seitz EK filter. *Eperythrozoon parvum* was repeatedly separated from *E. suis* by filtering the serum portion of blood known to contain both species (exper. 4, 6, 9, 11, and 12).

Attempted Transmission with Urine.—Table 2 summarizes the results obtained from injecting susceptible pigs with urine obtained at all stages of acute eperythrozoonosis. Urine was collected from living cases as it was naturally voided and, at autopsy, it was withdrawn from the bladder with a syringe and needle. In those cases in which unfiltered urine was used, a time interval of less than five minutes occurred between recovery of the urine and its injection. Inoculated animals were observed for periods of from thirty-three to fifty-eight days.

From the results obtained, it is concluded that neither eperythrozoa nor any other pathogenic agent is present in the urine of pigs affected with acute eperythrozoonosis. In one instance (pig 46), *E. suis* survived twenty-four hours in a mixture of blood and urine.

Chicken Embryo Studies.—Seven- and

8-day-old embryos were used in these studies, and each was injected in the yolk sac with 0.5 cc. quantities of material. In an initial experiment, serum from a carrier was filtered with a 9-lb. Mandler candle and injected into embryos. Following incubation periods of five, six, and seven days, the pooled yolk and sacs were diluted in saline and filtered with a 12-lb. Mandler. The pig that received this material (20 cc.) remained normal and negative for 156 days.

Included in table 3 are the results of attempts made to cultivate *E. suis* in serial passage in chicken embryos. Defibrinated blood from acute cases heavily infected with parasites was used in the first passage into yolk sacs. A separate source of blood was used in each cultivation attempt; none of the materials were filtered.

Eperythrozoon suis was recovered from embryos eight days following inoculation in the first passage, but not from embryos in the third and fifth serial transfers (a total of 15 and 25 days in embryos, respectively).

Infectivity for Animals Other than Swine.—In previous studies,[2] *E. suis* and *E. parvum* were shown by cross-inoculation methods to be distinctly separate species from *Eperythrozoon wenyoni* of cattle. Foote *et al.*[4] reported that a mild case of virus anemia developed in a splenectomized calf following the injection of serum filtrates from affected pigs. No attempt was made to transfer the infectious agent back to swine.

A splenectomized calf was injected intravenously with 5 cc. of unfiltered, defibrinated blood from a pig undergoing acute infection with *E. suis*. The calf remained normal during a period of 118 days' observation. After forty-five days of this time had expired, the animal was shown to be susceptible to *E. wenyoni* by the injection of bovine carrier blood. On the ninety-sixth day, 5 cc. of defibrinated blood was obtained from the calf and injected intravenously into a susceptible pig. No infectious agent was recovered and the pig remained normal for a total of eighty-five days.

Additional splenectomized animals that have proved refractory to injections of heavily infected blood are Eperythrozoon-susceptible mice, rats susceptible to *Haemobartonella muris* and carriers of the parasite, guinea pigs, and rabbits. No attempt was made to determine if latent infections were established in these animals.

Arsenical Therapy.—The specific parasiticidal action of neoarsphenamine against *E. suis* already has been reported.[8] Foote *et al.*[4] reported that the organic arsenical, aricyl, is very beneficial when 10 cc. of the drug is administered in early cases of virus anemia.

The results of experimental therapy with aricyl in acute eperythrozoonosis are given in table 4. The drug was found to be ineffective against either *E. suis* or *E. wenyoni*, while immediate control was obtained following the use of neoarsphenamine.

Data have been compiled on 55 acute experimental attacks of *E. suis* treated with neoarsphenamine. Single intravenous injections above 15 mg. per kilogram of body weight produced specific action in 48 instances and seven required a repeated dose

TABLE 2—Infectivity of Urine from Field and Experimental Cases of Acute Eperythrozoonosis

Pig injected No.	Quantity injected	Source of urine	Filtration	Results
32	8 cc.	Field case	N-Berkefeld	negative
44	8 cc.	Experimental pig 75	8-lb. Mandler	negative
32	4 cc.	Experimental pig BP*	unfiltered	negative
33	8 cc.	Experimental pig 30	unfiltered	negative
49	25 cc.	Field case*	unfiltered	negative
48	15 cc.	Experimental pig BT	unfiltered	negative
BW	8 cc.	Carrier pig 60	unfiltered	negative
46	0.5 cc. blood @ 20 cc. urine	Eperythrozoon negative pig BW	unfiltered	Acute eperythrozoonosis. Incubation period—15 days

*urine obtained at autopsy. @ defibrinated blood heavily infected with Eperythrozoon suis, mixed with urine and held twenty-four hours at 5 C. before injection.

TABLE 3—Attempted Cultivation of Eperythrozoon Suis in the Yolk Sac of Chicken Embryos

Pig injected No.	Quantity injected	Passage in chicken embryos	Results
BW	5 cc.	5th*	negative
43	5 cc.	3rd*	negative
45	5 cc.	1st @	Acute eperythrozoonosis. Incubation period—15 days

*eggs incubated five days before serial transfer of yolk and sac material. @ eggs incubated eight days.

to produce this action. Four animals died within forty-eight hours following therapy and all others recovered.

Neoarsphenamine administered prior to the appearance of clinical symptoms may prolong the incubation period. In 4 cases, the drug was given following the appearance of eperythrozoa in the blood of the inoculated pig but before clinical symptoms developed. Therapy caused a disappearance of the parasites; but relapses began usually in five to eight days, and these were controlled with additional injections. In this manner, the time until clinical symptoms developed in these 4 cases was prolonged for from twenty-five to forty-two days, the final attacks being untreated in order that the animals might be proved susceptible to acute infection. It is evident that the period of incubation might be extended indefinitely, provided that continuous microscopic examinations of the blood were made and each recidivation of *E. suis* given early therapy.

Eperythrozoon parvum has been found to be relatively resistant to neoarsphenamine in doses which are consistently effective against *E. suis*. Doses of from 15 to 30 mg. per kilogram are usually ineffective, while injections of 40 mg. per kilogram are effective irregularly. It has not been possible to establish a drug-fast strain of *E. suis*.

In the therapy of field cases, definite evidence of the value of neoarsphenamine in early treatment has been obtained. Fourteen cases received single, intravenous injections at doses ranging from 20 to 30 mg. per kilogram, and all recovered. *Eperythrozoon suis* was effectively removed in all on observations made twenty-four hours following therapy, and the temperatures had returned to normal. Marked clinical improvement was evidenced at this time or within an additional 24-hour period. In these same nine herds, 16 animals received no treatment. Eleven of these untreated pigs died and 5 recovered. It should be noted that none of the treated animals had evidenced clinical symptoms longer than seventy-two hours prior to receiving therapy. This is undoubtedly an important factor in securing recovery.

Additional drugs with specific action against *E. suis* include sodium arsenamide (caparside sodium, Abbott), p-arsonososuccinanilic acid (Sharp & Dohme), and sulfarsphenamine (Winthrop - Stearns, Inc.). The two former drugs have produced severe toxic reactions in pigs at therapeutic levels.

TABLE 4—Ineffectual Therapy of Acute Eperythrozoon Suis Infections with Aricyl

Pig No	Therapeutic agent	Dosage	Degree of Eperythrozoon infection	Results
97	Aricyl	16 mg./kg. (10 cc.)	very numerous	No effect upon *E. suis* Death occurred in 72 hours Lowest r.b.c. count—2,320,000
30	Aricyl	25 mg./kg. (10 cc.)	extremely numerous	No effect upon *E. suis* Death occurred in 24 hours Lowest r.b.c. count—3,220,000
28	Aricyl	100 mg./kg. (54 cc.)	very numerous	No effect upon *E. suis*. Evidence of overdosage in 24 hours, died on 5th day Lowest r.b.c. count—2,330,000
35	Aricyl	50 mg./kg. (15 cc.)	very numerous	*E. suis* remained unchanged for 48 hours.
	Aricyl	70 mg./kg. (21 cc.)	very numerous	No effect upon *E. suis* Death occurred in 72 hours Lowest r.b.c. count—2,940,000
76	Aricyl	14 mg./kg.	very numerous	*E. suis* remained unchanged for 48 hours
	Neoarsphenamine	30 mg./kg.	very numerous	Negative in 6 hours. Temperature reduced from 106.0 to 100.2 F. in 24 hours, when pig was again eating and active Lowest r.b.c. count—5,750,000
42	Aricyl	17 mg./kg. (20 cc.)	numerous	*E. suis* remained unchanged for 24 hours
	Neoarsphenamine	30 mg./kg.	numerous	Negative in 3 hours. Temperature reduced from 106.4 to 101.7 F. in 24 hours, and pig was noticeably improved Lowest r.b.c. count—4,320,000
Calf 322	Aricyl	6 mg./kg. (20 cc.)	*E. wenyoni* very numerous	*E. wenyoni* remained unchanged for 48 hours
	Neoarsphenamine	25 mg./kg.	*E. wenyoni* very numerous	Negative in 4 hours

Reproduction of the Disease in Unoperated Pigs.—Previous efforts to reproduce acute eperythrozoonosis in unoperated pigs had not been successful.[1] Foote *et al.*[4] reported that virus anemia could not be reproduced in unoperated swine.

Experiments were conducted following the premise that injections of enormous numbers of the infectious agent might be capable of overwhelming the defenses of the normally resistant animal. Unoperated pigs weighing from 15 to 30 lb. were injected intravenously with citrated blood heavily infected with *E. suis*. The amounts inoculated varied from 30 to 80 cc. Nine pigs were included in these studies. The presence or absence of latent eperythrozoon infection in these animals was not determined prior to their use.

Two pigs developed acute eperythrozoonosis and died on the sixth and ninth days following the blood injections. Erythrocyte counts dropped to lows of 3,700,000 and 2,270,000 cells per cubic millimeter. The course of the Eperythrozoon infection was identical to that noted previously in acute *E. suis* infections of field and splenectomized cases.[1, 3] Two additional pigs developed subclinical anemia following relatively heavy parasitic infections of short duration. Erythrocyte counts were reduced to lows of 4,720,000 and 4,900,000 cells per cubic millimeter. Two pigs developed increased temperatures only, which were associated with relatively heavy parasitic invasions of less than twenty-four hours' duration. Mild parasitic attacks resulted in 3 pigs, and these animals remained unaffected.

In all instances, *E. suis* was present in the blood of inoculated pigs immediately following the injections, usually in "occasional" numbers. In the clinical and subclinical cases, increased temperatures up to 106.3 F. were present, usually within twenty-four hours following the inoculations, when the parasites had multiplied in numbers sufficient to produce pathological effects.

DISCUSSION

It is recognized that these studies have not clarified the exact nature of Eperythrozoon organisms. However, it is evident that an ultramicroscopic agent with the properties attributed to the virus of Farley is not present in uncomplicated, acute eperythrozoonosis of swine.

Acute infections with *E. suis* may be differentiated from virus anemia by a number of important points. Those applicable to uncomplicated *E. suis* infections include the following: (1) The urine of acutely affected animals and carriers is not infectious. (2) Passage of blood serum through bacteria-tight filters at a negative pressure of 100 mm. of mercury or less usually removes the pathogenic agent. If passage of *E. suis* does occur, typical acute eperythrozoonosis results. (3) The disease may be halted immediately in the early stages by therapy with neoarsphenamine. Therapy during the period of incubation may prolong this period. Aricyl is of no apparent value as a specific agent. (4) *Eperythrozoon suis* will survive in heavily infected blood at—32 C. for at least thirty-one days (table 5). (5) Clinical and subclinical infections may be produced in unoperated pigs by the intravenous injection of large numbers of the parasites. (6) *Eperythrozoon suis* is innocuous in splenectomized calves.

It was observed in these studies that the presence or absence of eperythrozoa microscopically in the blood of the donor animal has an important bearing upon the infectivity of the serum and the Eperythrozoon species that may be recovered.

In two experiments (8 and 10, table 1) involving microscopically negative carrier donors, eperythrozoa could not be transmitted with unfiltered serum, although transmission occurred with whole blood. This is regarded as additional evidence that the Eperythrozoon structure is the infectious agent involved. The affinity of these parasites for the erythrocyte (as indicated in microscopic observation) would increase the probability of their recovery from whole blood rather than serum alone, partic-

TABLE 5—Preservation of Eperythrozoon Suis in Heavily Infected, Defibrinated Blood at —32 C.

Pig inoculated (No.)	Days stored	Quantity injected	Results	
			Prepatent period*	Incubation period@
49	1	12 cc.	3 days	6 days
BW†	1	8 cc.	4 days	7 days
42	5	5 cc.	4 days	8 days
37†	10	16 cc.	7 days	10 days
50†	10	8 cc.	3 days	6 days
201	31	8 cc.	3 days	6 days

†carrier of *Eperythrozoon parvum*. *time until first appearance of *Eperythrozoon suis* in the blood of inoculated animal. @time until first evidence of clinical symptoms following inoculation.

ularly when the organisms were very rare in the blood.

A total of 136 attacks of acute eperythrozoonosis have been studied in experimental swine. There has been no exception to the observation previously made[3] that eperythrozoa must be present in the blood of the affected animal in great numbers and for a sufficient time in order that acute symptoms may result. Acute infections reproduced in unoperated, experimental swine also conform to this statement.

The filterability of eperythrozoa, as noted in these studies, and the present inability to cultivate these parasites on cell-free mediums are acknowledged to be characteristics of the filterable viruses. However, to designate eperythrozoa as "reactionary bodies or artifacts" is not consistent with all of the scientific facts relating to these organisms, nor is it in agreement with the present method of classifying systemically these and related infectious agents.[7] On the basis of the present information, it can not be concluded that eperythrozoa are of viral origin, in view of the known filterability of other minute, cultivable, and noncultivable microorganisms and the close similarity which exists between Eperythrozoon and Haemobartonella organisms. Species of the latter genus have been cultivated on cell-free mediums.[7] Similarities between the two genera include the morphology and staining reaction, parasitic relation to the erythrocyte, the disease process and pathology, the activation of latent infections and removal of natural immunity by splenectomy, and the susceptibility to neoarsphenamine.

SUMMARY

1) Efforts to recover the "virus of Farley" from field and experimental cases of acute eperythrozoonosis were unsuccessful.

2) *Eperythrozoon suis* was passed through bacteria-tight filters in four of 29 individual serum filtrates. The parasite was completely removed from the same infectious serums by filters of finer porosity. It was found that at times only the cellular portion of the blood of carriers may be infectious.

3) *Eperythrozoon parvum* was found to be capable of traversing filters of fine porosity; however, most infections resulting from this parasite were innocuous.

4) No infectious agent could be recovered from the urine of pigs undergoing acute eperythrozoonosis.

5) Infection could not be established in a splenectomized calf, or in rats, mice, guinea pigs, or rabbits.

6) Eperythrozoa could not be maintained in chicken embryos by serial passage. In one experiment, *E. suis* survived eight days in the yolk sac. The parasite survived thirty-one days in frozen blood.

CONCLUSION

Acute eperythrozoonosis of swine is not identical to the condition described by Foote *et al.*[4] as "virus anemia." Full acceptance of "virus anemia of swine" as a disease entity awaits its reproduction consistently and entirely in the absence of eperythrozoa.

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A Study of the Pathology of the Intestine and Other Organs of Weanling Pigs When Fed a Ration of Natural Feedstuffs Low in Pantothenic Acid

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VITAMINS RECENTLY have assumed a position of great importance in swine nutrition, particularly with regard to the role of B vitamins in nutritional enteritis of young weanling pigs. Luecke *et al.*[1] and Cunha[2] stated that approximately 40 per cent of all pigs farrowed die before reaching market age. This is a tremendous loss to the swine industry. Most of this loss has been attributed to faulty nutrition. The purpose of this investigation was to study the gross and microscopic pathology of the intestine and other organs in weanling pigs when fed a ration of natural feedstuffs lows in pantothenic acid.

REVIEW OF LITERATURE

Kinsley[3] stated that "infectious necrotic enteritis" was widespread and second only to hog cholera in economic importance. The disease, usually designated as "filth disease," was thought to be caused by *Bacillus suipestifer*. McEwen[4] indicated that necrotic enteritis in swine was not always due to an infectious agent. The author reported necrotic enteritis in young pigs in a herd where swine fever was unknown and where Salmonella could not be detected. Selective mediums seeded with intestinal material failed to indicate the presence of Salmonella. Wintrobe and Mitchell[5] fed young pigs with an "adequate" ration containing "adequate" amounts of yeast. The yeast was gradually reduced and substituted by thiamine and riboflavin. This resulted in a severe degeneration of the posterior columns of the spinal cord, dorsal root ganglia, and the peripheral nerves. It was concluded that these changes were due to a deficiency of one or more components of the vitamin B complex, other than thiamine or riboflavin. Chick *et al.*[6] reported that at least two water-soluble factors contained in the filtrate and eluate fractions (B6) of Edgar and Macrae[7] were essential to the pig. Synthesis of pantothenic acid by Williams[8] made possible the direct evaluation of the role of this vitamin in nutrition. Wintrob *et al.*[9] noted diarrhea, marked impairment of growth, patchy alopecia, extensive colitis with abscess formation, ulceration, and atrophy of the cells lining the glands of the intestinal mucosa in pigs fed a synthetic diet deficient in pantothenic acid. Luecke *et al.*[1] investigated the role of nutrition in swine enteritis occurring in the field. Young pigs with diarrhea, without appreciable fever and fed a low protein, cereal grain ration with a lack of green forage, were given large doses of thiamine, riboflavin, calcium pantothenate, niacin, and pyridoxine intraperitoneally. These five vitamins, together with vitamin A and D, were incorporated in the ration. Sixty-nine out of 80 pigs were completely cured. Luecke *et al.*[10] produced pantothenic acid deficiency in pigs fed a basal ration made up largely of corn. In lots not supplemented by pantothenic acid, the animals showed diarrhea, followed by locomotor incoör-

TABLE I—Feed Mixture Used in Pantothenic Acid Studies

Ingredients		Composition	
Corn	74%	Crude protein	18%
Soybean-oil meal	23%	Pantothenic acid	4.53 mg./lb.
Complex mineral mixture	3%	Vitamin A	2000 I.U./lb.
		Vitamin D	200 I.U./lb.
		Niacin	10.00 mg./lb.
		Riboflavin	2.00 mg./lb.
		Vitamin B_{12}	6.25 mg./lb.

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dination. Microscopically, the sciatic nerve showed myelin degeneration. McMillen *et al.*[11] showed that a basal ration of corn, oats, expeller soybean-oil meal, meat scraps, alfalfa meal, and mineral mixture was deficient in pantothenic acid, nicotinic acid, and riboflavin for weanling pigs. When supplemented with these three vitamins, a significant increase in daily gain in weight and prevention of deficiency symptoms followed. Luecke *et al.*[12] found that weanling pigs fed a basal ration of corn, soybean and complex mineral mixture, supplemented with synthetic B vitamins, but containing no calcium pantothenate, produced severe symptoms of pantothenic acid deficiency.

MATERIAL AND METHODS

Hog cholera-immunized weanling Duroc-Jersey pigs approximately 8 weeks old were self-fed the basal ration employed by Luecke *et al.*,[12] with slight modifications as shown in table 1. The animals were housed in a concrete-floored barn and had access to clean water.

Eight control pigs received the same ration supplemented by 10.0 mg. per pound of calcium pantothenate. The pigs were weighed on the fourteenth, eighteenth, twentieth, and twenty-sirth day after being placed on the ration. On each of these days, 2 pigs on the deficient ration were bled for blood determinations, slaughtered, and the amount of food consumed was determined. At necropsy, sections of the small and large intestine, kidney, liver, and adrenals were removed and immediately fixed in Zenker's fluid. Paraffin sections, cut 4 to 5μ in thickness, were stained with hematoxylin-eosin.[13]

RESULTS

The daily average food consumption, gain in weight, and ratio of feed consumed per pound of weight gained are shown in table 2.

Average weights of the two lots of pigs during the period of investigation are illustrated in graph 1.

Clinical Picture.—Some of the animals showed loose feces after ten to twelve days, and by the fourteenth day all the pigs showed diarrhea. The animals were dull and developed anorexia. By the eighteenth day, a few blood flakes were detected in the fecal material. During the period of observation, there was no indication of alopecia nor of "goose stepping."

TABLE 2—Comparison of Pigs on the Control and Deficient Ration

Item	Control pigs	Pigs on deficient ration
Ave. initial weight	22	20
Ave. daily food consumption	1.99	1.52
Ave. daily gain in weight	0.68	0.31
Feed consumed per lb. of weight gained	2.94	4.86

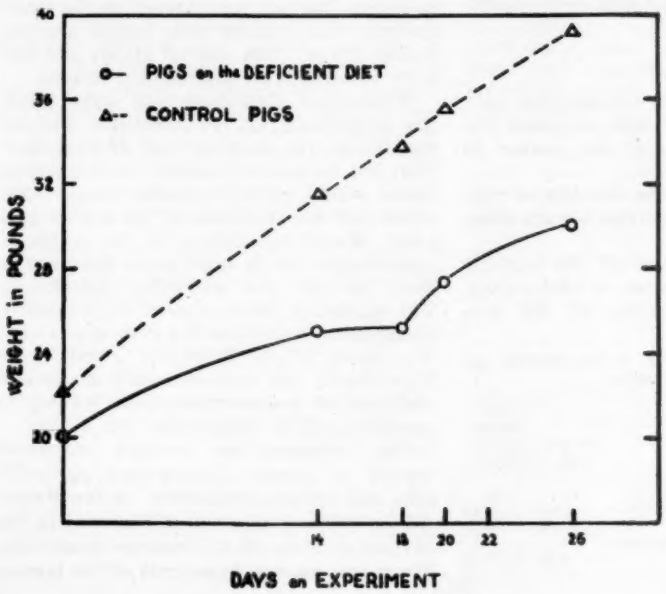
Blood Picture.—A mild normocytic anemia was present in some animals and anisocytosis was detected in 2 pigs. Leukocytosis was observed in all the deficient animals and was most marked on the eighteenth day, when the average leukocyte count was 32,770 per cubic millimeter of blood.

Gross Pathology.—The gross and microscopic alterations were confined mainly to the large intestine, although in the later stages of the disease the posterior part of the ileum was occasionally involved. In the early stages, the large intestine showed diffuse congestion, excessive discharge of mucus, and the intestinal wall was thick and edematous (fig. 1). Later, ecchymotic hemorrhages appeared, particularly in the tissue adjacent to the ileocecal valve. This was followed by the development of small round discrete ulcers approximately 0.5 cm. in diameter. Occasionally, fecal material adhered to the inflamed mucosa but could be washed off easily by a gentle stream of water. Lymph nodules in the wall of the intestine were hypertrophied and easily palpated. One pig, slaughtered on the twenty-sixth day, showed both healed and unhealed ulcers. The adrenal glands and kidneys presented no macroscopic lesions.

Microscopic Pathology.—In some areas, the epithelium of the intestinal mucous membrane was necrotic and desquamated. This led to the development of superficial ulcers which rarely extended deeper than about half the thickness of the lamina propria. Mucus accumulated in the crypts of Lieberkuhn, and in some areas appeared to flow out of the glandular epitehlium. The epithelial lining showed retrogressive changes and was distended with mucus (fig. 2). Some of the intestinal glands were hyperplastic and distended with mucus and degenerated polymorphonuclear leukocytes producing cystic dilatations (fig. 3).

The inflammatory exudate consisted mainly of mucus, degenerated epithelial cells, and necrotic leukocytes. A few *Balantidium coli* organisms were observed in the surface exudate of the mucous membrane. There was marked hyperemia of the lamina

Fig. 1—Large intestine showing congestion, edema, and thickening of the wall.



Graph 1—Average weights of pigs on control and pantothenic acid-deficient diet.

propria which developed into hemorrhagic foci. Some capillaries lying immediately below the surface epithelium were markedly distended with blood (fig. 2). The cellular reaction in the lamina propria was main-

Fig. 2—Section of large intestine showing desquamation of epithelium, exposed and congested blood vessel, edema, mucus-distended glands, and periglandular infiltration of leukocytes. Hematoxylin-eosin stain. x 190.

ly lymphocytic in nature, although a few neutrophils, plasma cells, and macrophages were detected. There was a marked periglandular infiltration of leukocytes.

The submucosa was congested and edematous with hyperplastic lymph nodules and a few macrophages in various stages of phagocytic activity.

One of the animals, destroyed on the twenty-sixth day, showed foci of regeneration as evidenced by the presence of fibroblasts (fig. 4). The number of crypts seen in these areas was relatively low and endothelial buds were evident. Columnar epithelium was seen bridging over the area of fibroblastic activity.

The capillaries of the adrenal cortex, particularly of the fascicular and reticular zones, were dilated and the parenchymatous cells appeared atrophied. The endothelial lining of the medullary sinusoids, as well as the adjoining parenchymatous cells, was degenerated and agglomerated in irregular masses in the remnants of the sinusoids.

The histopathological changes in the kidney were characterized by congestion of the medulla, which later developed into areas of diffuse hemorrhage. Shrinking of glomerular tufts and edema were found in 2 pigs (fig. 5).

Congestion, parenchymatous degeneration, and occasional interstitial edema were the principal histopathological changes in the liver.

Fig. 3—Section of large intestine showing cystic dilatation of glands resulting from hyperplasia and overdistention with mucus. Hematoxylin-eosin stain. x 50.

DISCUSSION

This investigation showed that a commonly used feed, consisting of corn, soybean-oil meal, and mineral mixture supplemented with niacin and riboflavin, produced deficiency symptoms which did not develop in control animals fed the same diet supplemented with pantothenic acid.

Pigs on the pantothenic acid-deficient ration showed a decrase in appetite, significant decrease in rate of weight gained, and inefficient utilization of feed. Diarrhea developed after about two weeks. The histopathological changes were generally confined to the large intestine. In contrast to the niacin deficiency reported by Dunne *et al.*,[14] the fecal material adhered to the intestinal wall as small discrete plaques and was washed off easily by a gentle stream of water. Wintrobe *et al.*[9] observed polymorphonuclear and mononuclear leukocytic infiltration in the periglandular interstitial tissue. In this study, polymorphonuclear cells infiltrating into the tissues were few in number. Morgan and Hawkins[15] and Hagen[16] reported that *B. coli* organisms were commonly present in the gut of pigs and apparently of little importance. In view of this, it was felt that the incidental presence of *B. coli* made no contribution to the production of lesions.

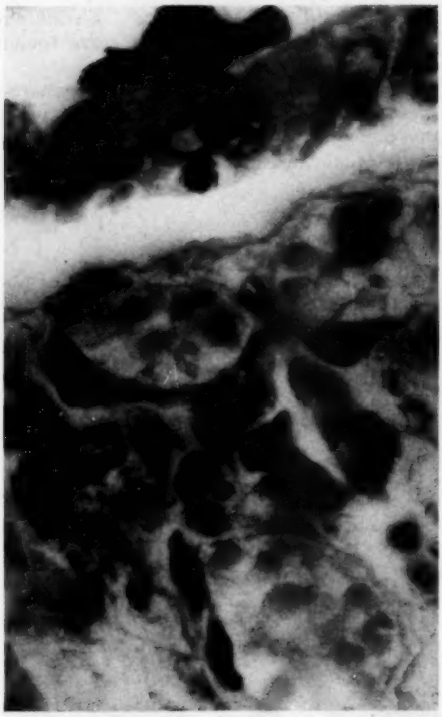


Fig. 4—Section of large intestine showing proliferation of fibroblasts and engorged capillaries in the subepithelial area. Hematoxylin-eosin stain. x 1150.

Williams *et al.*[17] suggested the possible role of pantothenic acid as a part of coenzyme A, in acetylation processes. These processes included the acetylation of choline and aromatic amines and the condensation of oxalacetate to form cis-aconitate. This disturbed the detoxification mechanism of the organism and led to a disturbed cellular physiology. In this instance, the disturbed physiology manifested itself as a colitis.

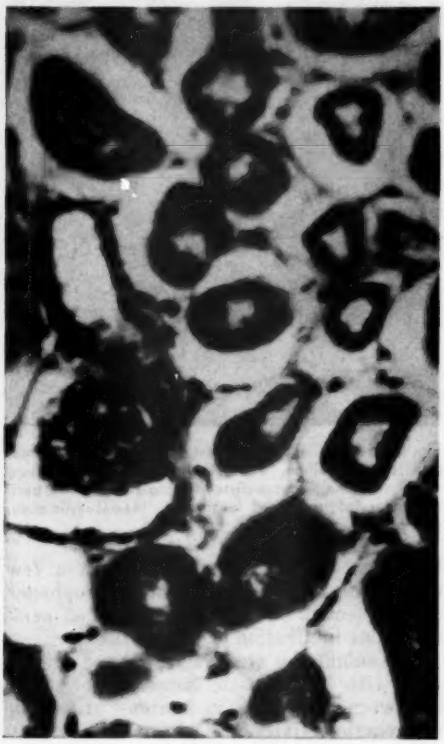


Fig. 5—Section of kidney showing edema of cortex and shrunken glomeruli. Hematoxylin-eosin stain. x 170.

SUMMARY

Eight Duroc-Jersey weanling pigs fed a ration of natural feedstuffs low in pantothenic acid showed a decrease in the rate of weight gained, inefficient utilization of feed consumed, and colitis accompanied by diarrhea.

The large intestine first showed degenerative changes and a few ecchymotic hemorrhages and, in the latter stages, small superficial discrete ulcers.

The columnar epithelium showed degenerative changes, and there was marked hyperemia of the lamina propria. The cellular reaction was mainly lymphocytic. The crypts of Lieberkuhn showed cystic dilatations and hyperplasia of the lymph nodules. One of the pigs slaughtered on the twenty-sixth day showed fibroplastic proliferation in the lamina propria.

Control pigs did not show the deficiency symptoms or lesions.

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The Value of Inclusion Bodies in the Diagnosis of Hog Cholera

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THE CRITERIA on which a positive diagnosis for hog cholera is based are inadequate in many respects. Inclusion bodies have been reported to be present in hog cholera and to be of diagnostic significance. It was the purpose of this study to do further research on the presence of inclusion bodies in cholera and to establish their place as a diagnostic aid.

Cholera first appeared in swine in the United States in 1833 and has become the major disease causing loss to swine raisers. Diagnosis based on case history, symptoms, and postmortem findings often fails to give positive diagnosis of cholera or to distinguish it from erysipelas, salmonellosis, and other diseases of swine.

To secure a positive diagnosis, it is often necessary to inoculate known susceptible swine with filtrates of tissue from diseased swine. With an incubation period of five to six days for cholera, this delays positive diagnosis of cholera until it is often of little practical value. This method is also expensive, since no small experimental animal can be used and the inoculated swine, when giving a positive test, may die of cholera.

REVIEW OF THE LITERATURE

Inclusion Bodies.—Cellular inclusions can be classified into three general groups: the naturally occurring, viral, and a third for which the author has found no satisfactory name. In this paper, it will be called nonviral. Maximow and Bloom[1] make reference to several normal cell constituents, calling them inclusion bodies. Pinkerton[2] lists normally appearing structures often confused with cytoplasmic and nuclear viral inclusions as ingested erythrocytes and nuclear lobes of neutrophils, large eosinophilic nucleoli, secretion granules, etc.

Viral inclusions occur only as the result of virus infection, although many virus diseases cause no inclusion body formation. These inclusions, as described by Pinkerton,[3] may occur in the nucleus, cytoplasm, or in both the nucleus and cytoplasm of the same cell. They may be eosinophilic or basophilic, depending on their nature and the staining procedure used. Their differentiation from normal cell constituents and nonviral inclusions is often difficult if not impossible. Cowdry[4] characterizes nuclear viral inclusions as usually being acidophilic, containing a clear halo between it and the nuclear membrane, and as having margination of the basophilic chromatin on the nuclear membrane. He also compares oxychromatic degeneration with inclusion body formation.[5]

In the third group, or nonviral, are included all those inclusions or inclusion-like bodies not normally found in the cell and not virus-caused. Inclusions of this group can be induced.[6,7]

Inclusion Bodies as Diagnostic Aids.—Inclusion bodies play a prominent role in the diagnosis of many virus diseases. Pinkerton,[8] in his discussion of 37 virus diseases of human beings, lists five diseases in which diagnosis is completely dependent on inclusion bodies.

In the field of animal diseases, the Negri body of rabies, reported by Negri in 1903, was for many years the only cellular inclusion of diagnostic importance. More recently, inclusion bodies have had importance in the diagnosis of pseudorabies,[9,10] canine distemper,[11-13] infectious canine hepatitis, [14-17] and feline panleukopenia.[18]

Inclusion Bodies of Hog Cholera.—Several extensive studies [19-23] have been made to determine the histological changes which accompany hog cholera, with almost complete agreement as to the changes which take place. The most important changes are in the vascular system, the first being damage to the endothelium of the smaller arteries and capillaries. The endothelial cells swell and may proliferate in tumor-like formations. The endothelium may be thrown off and enter the circulation producing embolism of smaller vessels. The walls of many of the vessels swell and show hyaline

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degeneration. These vessel changes are more pronounced in the lymph nodes, spleen, and kidneys, and are less often seen in the central nervous system, liver, intestinal tract, skin, and other organs. In acute cases, hyaline degeneration of vessels is prevalent; while in chronic cases, endothelial proliferation is more evident.

Himmelberger,[25] using Giemsa's stain, reported finding cytoplasmic inclusions in smears of the conjunctival epithelium of cholera hogs which were examined during the rise in body temperature.

Morris[24] made conjunctival scrapings of hogs, guinea pigs, rabbits, and monkeys which had been inoculated with the cholera virus. Scrapings made over a two-week period and stained with Giemsa stain failed to show inclusion bodies. The hogs which were used in the experiment showed typical hog cholera symptoms following inoculation, but the guinea pigs, rabbits, and monkeys showed no evidence of disease.

In observations of nerve tissues of 39 cholera hogs infected with four strains of virus and 7 normal hogs, Seifried[35] failed to find typical inclusion bodies in the cholera-infected hogs. In a small percentage of cases, there were found in the nucleus of nerve cells inclusion-like bodies which were considered products of nuclear degeneration rather than true inclusions.

Muir[26] injected mice, hamsters, ferrets, white rats, guinea pigs, and rabbits with tissue from cholera hogs, in an attempt to find small experimental animals for use in the diagnosis of hog cholera. These animals failed to show any clinical symptoms or pathology of diagnostic value.

Boynton *et al.*,[37] in examination of smears from the gall bladders of 600 cholera-infected hogs and 50 hogs suffering from other diseases, reported intranuclear inclusion bodies in the cholera-infected hogs. Following a modified Kingsley's[38] staining method, the inclusions stained a deep magenta and were easily differentiated from the nucleoli which stained a light blue.

Sippel,[29] in commenting on Boynton's method states:

> The three things then, the sharply outlined and stained nuclei, the granular cytoplasm, and the inclusion bodies, make the identification of a positive slide relatively simple. In negative cases the cells will stain indistinctly, or not at all. Those that do take the stain have a basket-like appearance and poorly defined margins.

He further states that the more advanced cases show the most characteristic slides, but that that there is insufficient data on other virus diseases of swine to know their effect on this type of smear.

Plummer[30] cut paraffin sections from the gall bladders of 38 cholera-infected hogs and stained them with hematoxylin and Le Mesurier's triple eosin mixture. The epithelium of the musoca was eroded from 23 animals. In the remaining 15, no inclusion bodies were found in the epithelial cells nor were there any other cell changes of diagnostic value.

Materials and Methods

The tissues selected for histological sections were the urinary bladder, gall bladder, tonsil, liver, spleen, kidney, ureter, trachea, and spinal cord. The gall bladder sections were taken from the area adjoining the cystic duct. The kidney sections included the entire cross section from cortex to pelvis. Trachea and spinal cord sections were taken from the area of the sixth cervical vertebra. Gall bladder smears were made from the area adjoining the cystic duct.

The tissues and gall bladder smears were taken from both normal and diseased hogs. Thirteen normal hogs were used. Three of these were selected from swine at the research farm of the Missouri School of Veterinary Medicine, and 10 were selected from hogs being killed at a meat-packing plant.

For diseased hogs, tissues and gall bladder smears were taken from 24 virus hogs of the Jensen-Salsbery Laboratories, Inc., which were being killed on the sixth day following virus inoculation. Thirty-three diseased hogs were selected from the postmortem laboratory of the veterinary school. Sixteen of these hogs had been diagnosed as having cholera, 9 pneumonia, 1 necrotic rhinitis, 6 necrotic enteritis, and 1 atropic rhinitis. The cholera-infected hogs were in various stages of the disease, but no tissues were taken from hogs which were dead when presented to the postmorten laboratory for diagnosis.

The tissues were taken from the hogs within thirty minutes after death and put in the fixative. Zenker's fluid was used for the 3 normal hogs from the research farm and for 5 of the virus hogs from Jensen-Salsbery Laboratories. The tissues were left in Zenker's fluid for twenty-four hours, then washed in running water overnight, and stored in 80 per cent alcohol until imbedding. The tissues from the remaining hogs were all fixed in 10 per cent formalin, in which they were stored until imbedding. The tissues were imbedded in paraffin by the standard chloroform method. Sections from these were cut 7μ thick, floated from a 45 C. water bath onto slides, and allowed to air-dry. They then were put into the 56 C. paraffin oven for thirty minutes and stored until staining.

Three staining methods were used for the tissue sections: Kingsley's, Harris's hematoxylin and eosin, and Harris's hematoxylin and eosin with phosphotungstic acid.

The gall bladder smears were made from the fixed stored tissues. The excess mucus was removed from the surface with a piece of

gauze. The mucous membrane was then scraped lightly with a knife and the cells transferred to a clean slide and spread with the aid of a knife. The cells did not scatter over the slide as well as they do when smears are made from fresh tissue. The slides were air-dried, then put in absolute methyl alcohol for fifteen minutes, again air-dried, and stored until staining. Staining was done with Harris's hematoxylin and eosin, following the procedure used for the sections, and Kingsley's stain.

DISCUSSION AND RESULTS

The ease of use and the results of the various staining techniques varied widely. For the sections, Harris's hematoxylin and eosin was the easiest stain to control and gave the best slides. Kingsley's stain, besides being more time-consuming in preparation, was not an easy stain to use. With it all, timing needed to be exact and the staining solutions required frequent changing. In the staining of smears, Harris's hematoxylin and eosin again gave the best results. Kingsley's stain on the smears, as with the sections, was difficult to use and gave poor results. Giemsa's stain was tried on several smears but was discarded because it proved inferior to the other stains.

Table 1 shows the tissues taken from the various normal and diseased hogs and the staining methods used for them. All the tissue sections and smears were examined under low power, high power, and oil-immersion objectives. Nothing was found on any of the slides which the author believes at the present status of this study could be classed as an inclusion body. However, several tissues showed points of interest worthy of discussion.

In tissue sections, the trachea, urinary bladder, and gall bladder slides showed nothing of significance. In the large motor neurons of many of the spinal cord slides, from both the normal and the cholera-infected hogs, one to four nuclear eosinophilic bodies 1 to 3μ in diameter were found. They are possibly the same as the inclusion-like bodies reported by Seifried.[31]

TABLE I—Summary of Tissues and Stains Used

Tissue and physical condition of hog	No. tissues and staining method		
	Harris's hematoxylin and eosin stain	Kingsley's stain	Harris's hematoxylin and eosin with phosphotungstic acid stain
Tonsil			
Normal hogs from research farm	3	3	..
Cholera-infected hogs from serum plant	5	5	..
Trachea			
Normal hogs from research farm	3	..	..
Cholera-infected hogs from serum plant	5	..	..
Kidney			
Normal hogs from research farm	3	3	..
Cholera-infected hogs from serum plant	5	5	..
Spleen			
Normal hogs from research farm	3	3	..
Cholera-infected hogs from serum plant	5	5	..
Ureter			
Normal hogs from research farm	3	3	..
Cholera-infected hogs from serum plant	5	5	..
Spinal cord			
Normal hogs from research farm	3	3	3
Normal hogs from packing plant	5	5	..
Cholera-infected hogs from serum plant	24	24	19
Cholera-infected hogs from postmortem laboratory	3	..	..
Bladder			
Normal hogs from research farm	3	3	..
Normal hogs from packing plant	5	..	..
Cholera-infected hogs from serum plant	5	22	18
Cholera-infected hogs from postmortem laboratory	2	..	..
Liver			
Normal hogs from research farm	3	3	3
Normal hogs from packing plant	4	..	..
Cholera-infected hogs from serum plant	17	17	17
Cholera-infected hogs from postmortem laboratory	4	..	..
Gall Bladder			
Normal hogs from research farm	3	3	3
Normal hogs from packing plant	4	..	..
Cholera-infected hogs from serum plant	17	17	17
Cholera-infected hogs from postmortem laboratory	4	..	..

In the cholera-infected hogs, a few of the livers showed small areas of coagulation necrosis. The spleens and tonsils also showed areas of coagulation necrosis, some of which were extensive. These lesions are commonly found in hog cholera.

The epithelium of the ureter, kidney-pelvis, and tonsil of the cholera-infected hogs showed increased granulation of the nucleus as compared to that of the normal hogs. Although this was readily observed on many of the slides, no diagnostic significance is given to it at the present time.

After examining the gall bladder smears from a number of animals, the slides were divided into three groups. Those smears whose cells took the stain well and had a distinct staining nuclear membrane and a granular nucleus were called positive. Those which did not take the stain well and which contained an indistinct staining nuclear membrane and a less granular nucleus were called negative. Following this method of classification, the slides were recorded as positive, negative, or undetermined. This classification is summarized in table 2. Figures 1 and 2 illustrate positive and negative slides.

It will be observed from table 2 that none of the normal hogs gave positive smears. The smears from the 57 diseased hogs

TABLE 2—Summary of Findings on Gall Bladder Smears

	Positive	Negative	Uncertain	Total
Smears stained with Harris's hematoxylin and eosin stain				
Normal hogs from research farm	..	3	..	3
Normal hogh from packing plant	..	10	..	10
Cholera-infected hogs from serum plant	24	..	..	24
Cholera-infected hogs from postmortem laboratory	15	1	..	16
Pneumonia hogs from postmortem laboratory	8	1	..	9
Necrotic rhinitis hog from postmortem laboratory	1	..	..	1
Necrotic enteritis hog from postmortem laboratory	6	..	..	6
Atrophic rhinitis from postmortem laboratory	1	..	..	1
Smears stained with Kingsley's stain				
Normal hogs from packing plant	..	4	1	5
Cholera-infected hogs from serum plant	18	1	..	19
Cholera-infected hogs from postmortem laboratory	1	2	1	4

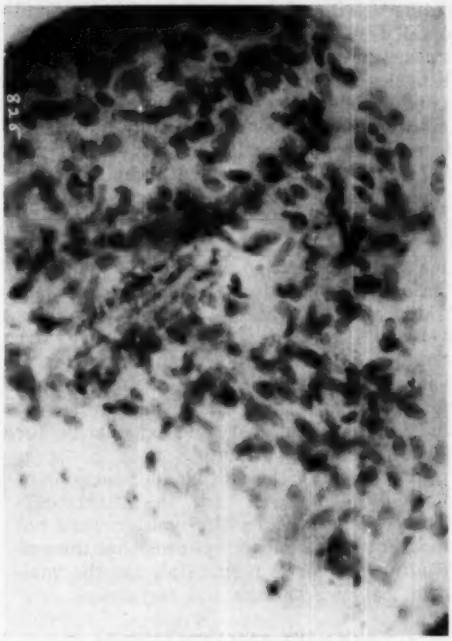

Fig. 1—Gall bladder smear classed as positive.

Fig. 2—Gall bladder smear classed as negative.

stained with Harris's hematoxylin and eosin were all positive but two, and the 23 smears from diseased hogs stained with Kingsley's stain were all positive but four. This clearly shows that the gall bladder smears of diseased hogs usually stain differently from normal hogs, but it does not differentiate those hogs with cholera from other diseased hogs and is, therefore, of no diagnostic value. When the gall bladders of the diseased hogs were opened on postmortem examination, they were found to contain a more viscous and more concentrated bile than those of normal hogs. This would be expected, since diseased hogs are usually off feed. This greater than normal concentration of the bile in the diseased hogs may affect the gall bladder mucosa, and is offered as a possible explanation for the difference in staining.

In comparing the gall bladder sections of normal and diseased hogs, the marked differences observed on the smears were not apparent. The author believes that the embedding process is responsible for the variation between smears and sections.

SUMMARY AND CONCLUSIONS

1) Inclusion bodies were not found in any of the tissues studied and, therefore, were not considered to be of any value in diagnosing hog cholera.

2) Gall bladder smears of diseased hogs showed cell changes not found in normal hogs.

3) The cell changes observed were not specific for hog cholera; therefore they could not be used as a diagnostic aid.

4) More cellular detail was observed in smears from the gall bladder epithelium than in sections, although the same staining techniques were used.

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Further Observations on the Neuropathological Diagnosis of Hog Cholera

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IN A PREVIOUS communication,[6] literature was reviewed with respect to the applicability of neuropathological examinations to the diagnosis of hog cholera. The findings on 124 diseased and normal porcine brains, collected over a period of ten years, were presented. The majority of 31 uncomplicated and 13 complicated field cases of hog cholera was found to be associated with a disseminated nonpurulent encephalitis, characterized primarily by proliferation of mesodermal cellular elements. Twenty-four of 35 normal swine, varying in age from 6 to 360 days, presented subventricular aggregates of ectodermal glia cells which decreased in number and intensity with age and which apparently were of no pathological significance. The occurrence of such "normal" aggregates was not clearly specified in the earlier literature and was probably a contributing factor to the divergence of opinion on the neuropathological diagnosis of hog cholera. Among 22 cases of infectious diseases other than hog cholera and 23 cases of noninfectious disorders, a total of 4 cases showed small, usually single, neurological alterations.

On the basis of this study, the opinion was expressed that the neuropathological examination of suspected hog cholera cases constituted a valuable diagnostic aid, especially if other neurological entities such as listeriosis, Aujeszky's disease, infectious encephalomyelitis (Teschen disease), and rabies could reasonably be ruled out.

The source material for the above work had been drawn from an area of limited hog production, largely dependent upon garbage feeding.

In a preliminary report on the relationship of swine encephalitis to hog cholera, Jones and Doyle[9] confirmed the occurrence of encephalitic lesions in the majority of hog cholera cases in an area of major hog production. However, their findings of such alterations in one-fourth of non-hog cholera cases and in more than one-third of healthy hog cholera-vaccinated cases lead the authors to caution against reliance upon neuropathological diagnosis in hog cholera.

The purpose of the present study was to compare the character and incidence of encephalitic lesions in additional hog cholera cases with those occasionally seen in non-hog cholera cases and in controls, especially from hog cholera-free areas (Canada). Another objective of this study was to test the statistical significance of encephalitic lesions in the diagnosis of hog cholera.

MATERIALS AND METHODS

The technical procedures in the present study were essentially comparable to those previously reported.[6] In five attempts, it was possible to confirm the neuropathological diagnosis of hog cholera by animal-transmission experiments. The present specimen material consisted of 35 cases of hog cholera, 20 vaccinated by various methods, 4 with definite neurological diseases, and 8 with infectious diseases other than hog cholera, and 52 cases of noninfectious diseases. The controls consisted of 36 non-hog cholera-vaccinated animals. Two of them came from Connecticut and 34 from Canada—the latter obtained through the courtesy of Drs. R. Gwatkin, Hull, Quebec, and F. W. Schofield, Guelph, Ontario. Six brain specimens from diseased animals, which were submitted by Dr. Schofield and were representative of gastroenteritis, pasteurellosis, rhinitis, and experimental poisonings, were included in the general account.

NEUROPATHOLOGICAL OBSERVATIONS

As pointed out in the previous report,[6] encephalitic changes have been described, aside from hog cholera, in Aujeszky's disease, rabies, infectious encephalomyelitis, and listeriosis. For differential diagnosis, geographical pathology should largely be relied upon, in conjunction with pathogenicity tests and bacteriological studies. Diagnosis should rest upon recognition of the character and the distribution of the encephalitic changes, because individual foci,

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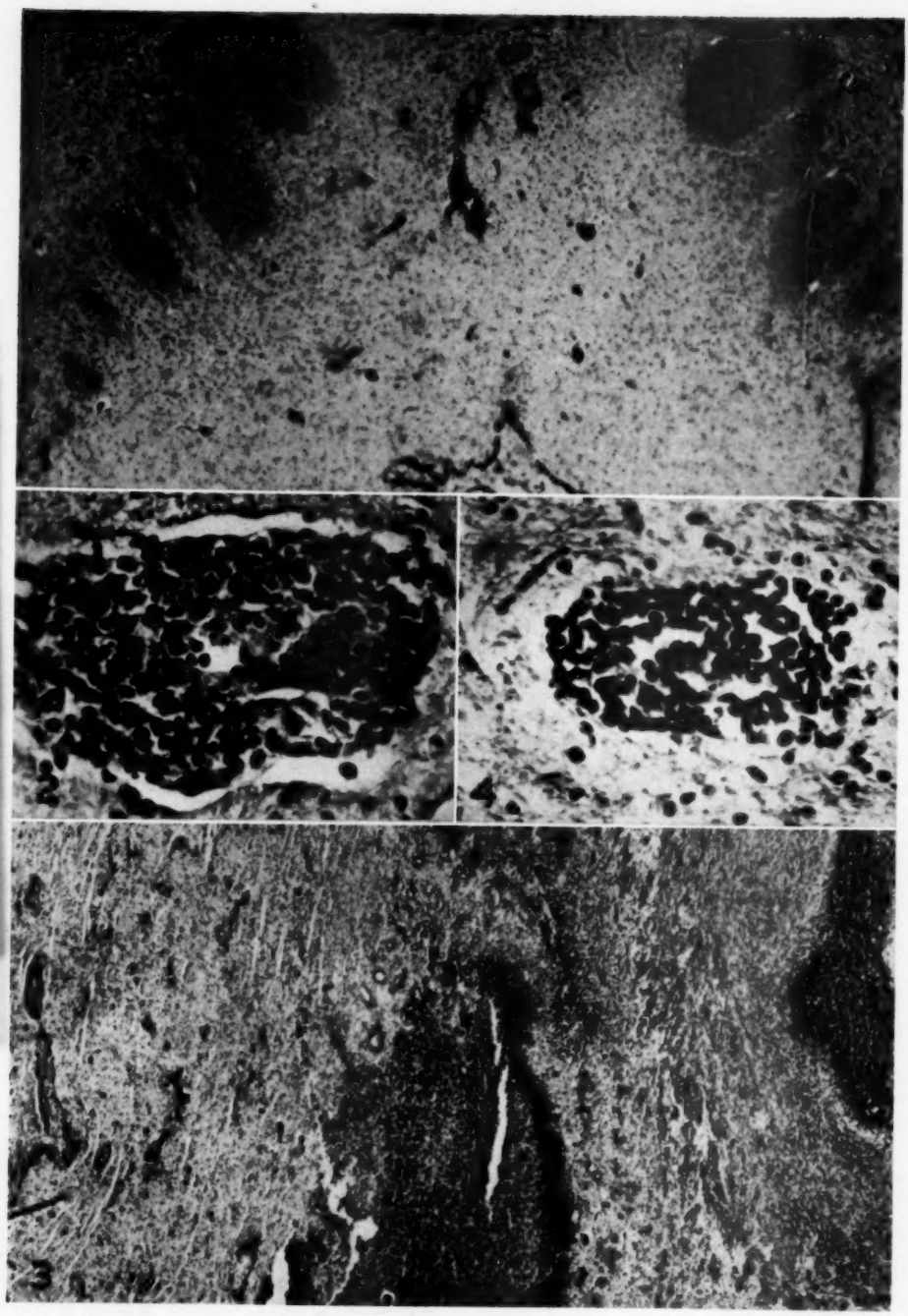

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regardless of their cause, may present difficulty in interpretation. On the whole, porcine infectious diseases other than those of recognized neurotropic propensities are not known to cause encephalitic alterations. Thus, the finding of encephalitic changes in bacteriologically proved cases of Salmonella infections would be more indicative of complicated hog cholera than of neurotropic properties of salmonellosis. A primary viral agent can not be ruled out entirely by failure to isolate a virus, since isolation is known to be difficult in the advanced stage of a disease. On the other hand, every effort should be made to establish possible neuropathological effects of uncomplicated infectious entities.

Hog Cholera.—The basic changes in the central nervous system, reflecting those in the visceral organs, were adequately described by Seifried and Cain.[14] The capillaries and precapillaries showed swelling and proliferation of the endothelium, followed by retrogressive changes to the point of necrosis. The changes considered to be the most striking by Seifried[12, 13] consisted of vascular and perivascular mononuclear infiltrates of the small blood vessels. The changes were found to be more or less equally distributed over the gray and white matter of the entire brain.

In the present material, the basic lesions conformed to the description in the literature. These are illustrated in fig. 1 and 2. Endothelial involvement was brought out under high power (fig. 2) and was considered of differential diagnostic importance, especially with respect to localized reactive foci (fig. 4) and nonspecific foci (fig. 11, 13, 15) of unknown etiology. On the whole, the disseminated character of the lesions proved to be of diagnostic assistance. The intensity of the lesions, however, seemed to increase with the duration of the disease.

Experimentally transmitted cases showed mild but disseminated lesions, primarily involving the endothelium, after destroying of the animals at the fastigium of the fever curve (usually on the seventh day postinoculation).

Two hyperimmunized weanling pigs from an immune sow, which were injected four times at two-week intervals with 3 ml. of commercial hog cholera virus and which were killed six days after the last inoculation, failed to exhibit encephalitic lesions. A litter mate control which was inoculated with the same virus at the time of the last inoculation succumbed to acute hog cholera and presented typical gross and neurohistological changes.

Fifteen pigs vaccinated by the simultaneous method likewise failed to exhibit neuropathological changes when examined one month or more following vaccination.

Eight pigs vaccinated with modified live hog cholera virus of rabbit origin (rovac, Lederle)* and killed seventeen days postvaccination proved to be normal on neuropathological examination. Ten pigs similarly vaccinated but challenged with virus of proved virulence fourteen days postvaccination were killed after an additional fourteen days. One of them presented in the thalamus a single paravascular infiltrative focus which had no resemblance, either in character or distribution, to typical hog cholera encephalitic foci (fig. 10 and 11).

Of 35 pigs immunized against hog cholera by various means, only 1 showed a nonspecific "encephalitic" focus. Thus, there was no evidence that live virus vaccination ordinarily caused an encephalitic picture similar to that in the spontaneous disease. Conversely, finding of such lesions in vaccinated pigs would be suggestive of vaccination failure or inapparent hog cholera infection.

*These porcine brains were received as fixed preparations through the courtesy of the Animal Industry Section, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Description of Figures on Opposite Page

PLATE I

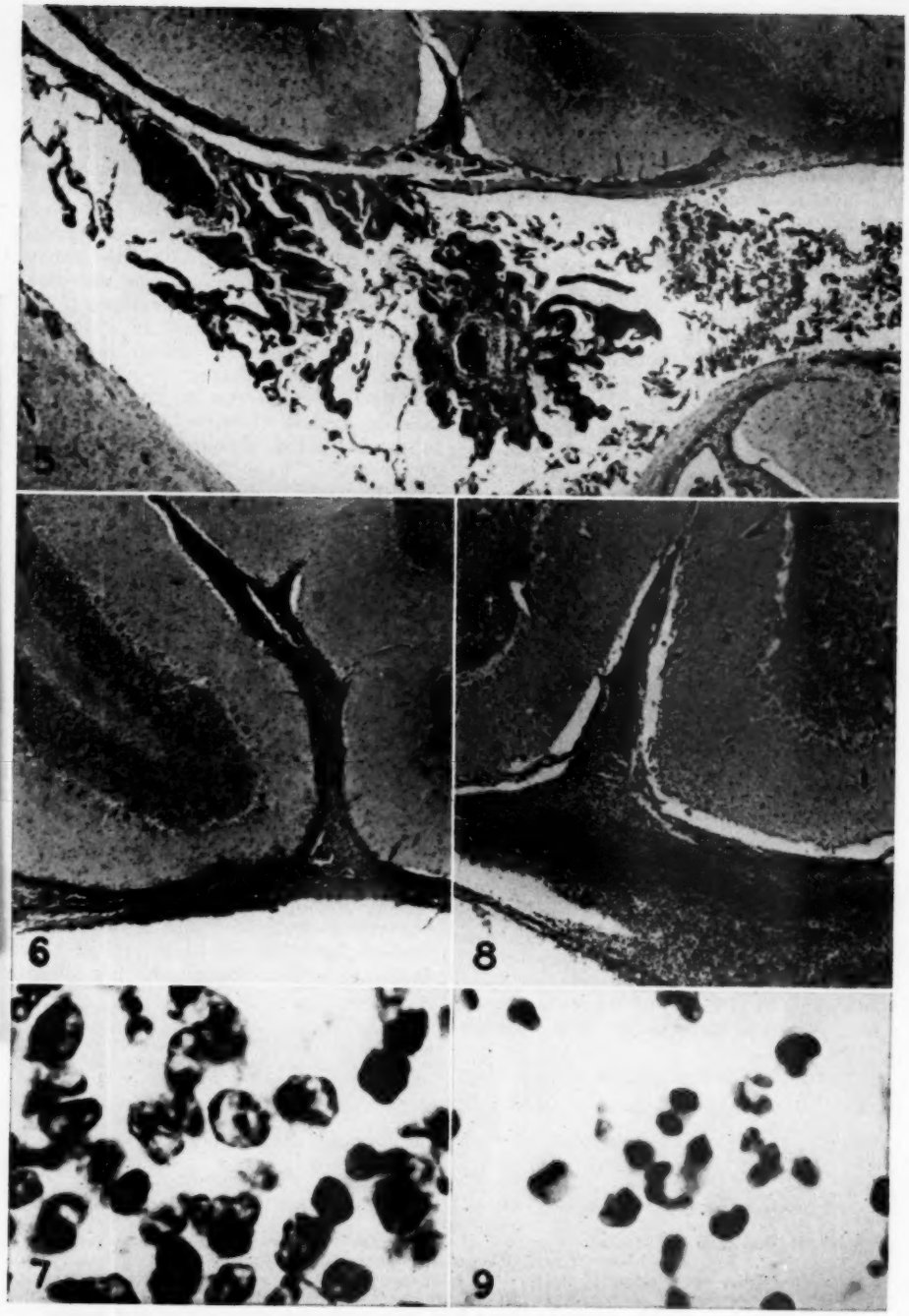
All figures are photomicrographs of porcine brain sections, embedded in paraffin and stained with hematoxylin-triosin.

Fig. 1—Hog cholera. Diencephalon at level of optic recess. Above recess are numerous vascular cuffs and a few glia foci. Disseminated nonpurulent encephalitis. x 40.

Fig. 2—Higher magnification of fig. 1. Thick infiltration of vascular wall with mononuclear cells. Right lower quadrant of vascular lumen shows hypertrophy and hyperplasia of endothelial cells. x 337.

Fig. 3—Listeriosis. Medulla oblongata shows a large central and a lateral focus of purulent material. The latter focus is delimitated by a fibrous capsule. The adjacent tissue shows marked reactive perivascular infiltrates. x 40.

Fig. 4—Higher magnification of fig. 3. Reactive perivascular cuff of mononuclear cells without endothelial involvement. x 337.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Listeriosis.—In the neuropathological description of this condition, Biester and Schwarte[1] noted the perivascular infiltrates of mononuclear cells and the occurrence of neutrophils. In the studies at the Storrs laboratory,[4] the lesions in listeriosis appeared conspicuously localized in the posterior brain stem and the cerebellar nuclei. An unusual accumulation of purulent material in the medulla was described recently by Helmboldt *et al.*[5] of this laboratory. In the latter case, some of the purulent foci presented a cone-shaped outline, while others were limited by a fibrous capsule (fig. 3). The adjacent tissue showed abundant cuffs of perivascular mononuclear infiltrates with occasional neutrophil leukocytes. The reactive foci, however, occurred in the vicinity of the areas of tissue destruction and were not indicative of a disseminated encephalitis. Furthermore, they failed to exhibit endothelial involvement (fig. 4), thereby furnishing a further distinguishing characteristic from hog cholera encephalitic lesions.

A Condition Resembling Lymphocytic Choriomeningitis. — According to Olitsky and Casals,[11] lymphocytic choriomeningitis is an endemic viral infection of lower animals, especially of the mouse, characterized by marked lymphocytic infiltration of the meninges, ependyma, and choroid plexuses.

This pathological picture has been seen at the Storrs laboratory in dogs on several occasions. Two dead Chester White Crosses, 10 weeks old and not hog cholera-vaccinated, were submitted from a penal institutional herd consisting of 160 pigs, 10 to 52 weeks old. The specimens came from a litter of 10, 4 of which had been destroyed after presenting severe symptoms of shivering and convulsion for twenty-four hours. Gross pathological examination failed to reveal any abnormalities except an induced skull fracture and slight adherent feces in the cecum. Microscopically, the visceral changes were limited to mucosal ulcers in the ileocecal valve, commonly associated with niacin deficiency.[2] The brain presented marked lymphocytic and mononuclear (fig. 7) infiltration of the leptomeninges (fig. 6); of the choroid plexuses, particularly of the fourth ventricle (fig. 5); and, to a lesser extent, of the ependyma of the lateral ventricles. Hog cholera could be excluded on pathological grounds. Since lack of further cases prevented follow-up studies, the condition was pathologically classified as lymphocytic choriomeningitis.

Purulent Meningitis.—In a small, garbage-fed herd of 32, varying in age from one to 52 weeks, 2, 10-week-old pigs of a litter of 12 showed staggering, paresis of the hindquarters, coughing, and rectal temperatures up to 108 F. for four days. This litter had been vaccinated for hog cholera by the simultaneous method fourteen days prior to the onset of the present illness. During the preweaning period, the owner had observed cases of omphalitis.

One of the affected pigs was submitted in a live condition, and it presented, aside from the previously mentioned symptoms, a hemoglobin reading of 12.5 Gm. per 100 ml. of blood (Haden-Hauser) and a leukocyte count of 19,850 per cubic millimeter, with 67 per cent lymphocytes. Toxicological examination by the Connecticut Agricultural Experiment Station revealed low values for lead and copper, and 75 parts per million of zinc, the latter probably being insignificant. Gross and microscopic examination failed to give a satisfactory explanation of the clinical picture, but was noteworthy in that the brain was without change eighteen days following simultaneous hog cholera vaccination.

The dead pig showed hemorrhagic material around the mouth and anus and thick fibrinous exudate over the pleura

Description of Figures on Opposite Page

PLATE 2

All figures are photomicrographs of porcine brain sections, embedded in paraffin and stained with hematoxylin-triosin.

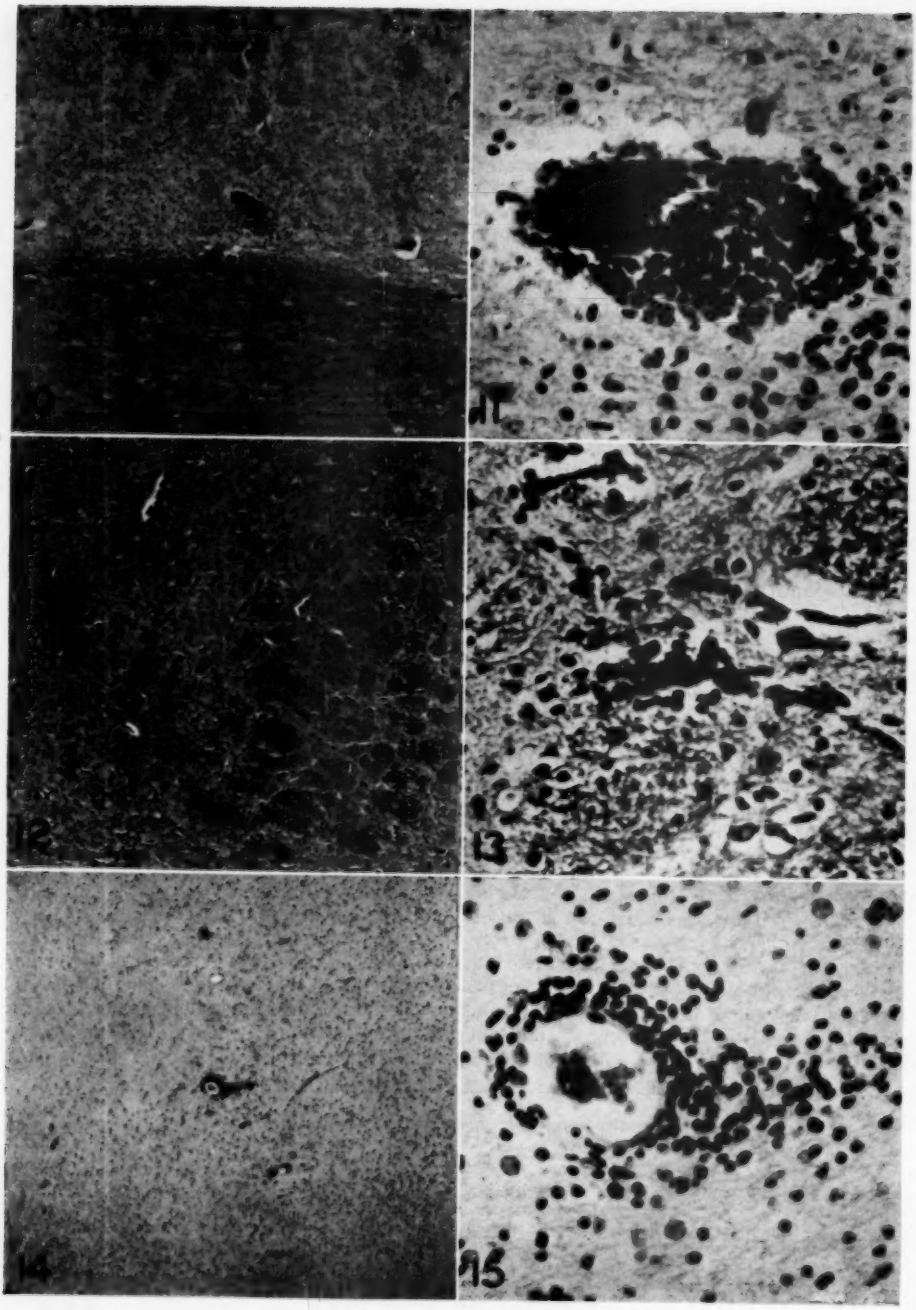
Fig. 5—Condition resembling lymphocytic choriomeningitis. Fourth ventricle shows marked thickening and lymphocytic infiltration of choroid plexus. The leptomeningi of the overlying cerebellum are similarly involved. x 40.

Fig. 6—Same specimen as fig. 5. Cerebellum presents marked lymphocytic infiltration of leptomeningi extending into interfoliar spaces. x 40.

Fig. 7—Higher magnification of fig. 6. Infiltrate is composed of lymphocytes and mononuclear cells. x 1518.

Fig. 8—Purulent meningitis complicating pleuritis. Cerebellum exhibits intense neutrophilic infiltration of leptomeningi. x 40.

Fig. 9—Higher magnification of fig. 8. Leptomeningeal infiltrate is shown to consist of neutrophils. x 1518.



causing collapse of the right lung. The microscopic changes consisted of serous pericarditis, fibrinous pleuritis, accompanied by atelectasis in the right lung, and cystic degeneration in the cecal submucosa. The entire brain exhibited severe meningeal infiltration (fig. 8) essentially composed of neutrophilic elements (fig. 9). As in the companion specimen, the brain substance was not involved except for slight subpial glia reaction. It was felt that the purulent meningitis in this case was of visceral metastatic origin, and that there was no evidence of hog cholera in spite of the suggestive clinical history.

Nonencephalitic Diseases and Controls.—Among 32 cases of infectious diseases other than hog cholera, representing such entities as erysipelas, salmonellosis, and tuberculosis, only one glia focus could be found in all the brain sections.

In 67 brain specimens representing nutritional disorders and poisonings, four exhibited isolated foci. In a case of suspected parathion (American Cyanamid Co.) poisoning, one of eight brains showed a small, single focus (fig. 12) which, under high power, resolved itself into loosely arranged glia cells without relation to a vascular structure (fig. 13).

Of 56 controls which had not been vaccinated for hog cholera, two brain specimens disclosed one focus each. Both of them occurred in the Canadian material which was obtained from mature slaughterhouse hogs. The foci were asymmetric accumulations in the vascular perimeter (fig. 14) of glia cells (fig. 15) which, in part, extended into the matrix.

Although the individual cellular foci in nonencephalitic diseases and in controls may have a superficial resemblance to those in hog cholera encephalitis, their minuteness and isolated occurrence, lacking definite vascular orientation and endothelial involvement, would preclude their interpretation as disseminated encephalitis.

Comparison with Encephalitides Other Than Hog Cholera.—As pointed out previously,[6] in utilizing disseminated encephalitic lesions in the diagnosis of hog cholera, other known encephalitides occurring in swine should be ruled out.

Although Aujeszky's disease has been described as causing lesions similar to those of hog cholera, its regional occurrence, clinical picture, and transmissibility to rabbits and mice serve as differential criteria.

The encephalitic lesions of rabies are likewise of a nonpurulent disseminated character but tend to extend into the gasserian ganglion and ganglion nodosum,[7] and tend to be accompanied by specific cytoplasmic inclusions, known as Negri bodies. In addition, the clinical picture and transmissibility contribute to the differential diagnosis.

Porcine encephalomyelitis (Teschen disease) has not been reported on this continent. Although Testi[15] considered the disease neuropathologically similar to hog cholera, Menzani[10] believed the lack of encephalitic changes in the corpora quadrigemina to be of differential importance, a view now concurred in by Horstmann *et al.*[8] According to the latter authors, the brain lesions were more severe than in human poliomyelitis (and in hog cholera, according to limited observations by the present authors) and lacked neutrophils in the cellular reaction. The widespread lesions resembled those found in Japanese B encephalitis and in other virus diseases, except in the spinal cord where they assumed the pattern of anterior poliomyelitis.

Thus, it is obvious that not every encephalitic change in porcine brains can be interpreted as hog cholera. Furthermore with increased experience, new en-

Description of Figures on Opposite Page

PLATE 3

All figures are photomicrographs of porcine brain sections, embedded in paraffin and stained with hematoxylin-triosin.

Fig. 10—Hog cholera-vaccinated and challenged. In ventral portion of thalamus, a single paravascular focus (in center) considered nonspecific. x 40.

Fig. 11—Higher magnification of fig. 10. Paravascular asymmetric infiltrate of mononuclear cells, flanked on lower right by loose glia cells. x 337.

Fig. 12—Parathion poisoning. In thalamus, a single loose glia focus (in center) considered nonspecific. x 40.

Fig. 13—Higher magnification of fig. 12. Loose glia focus without vascular orientation. x 337.

Fig. 14—Nonvaccinated control from Canada. In thalamus, a single vascular infiltrate (in center) with tail-like extension, considered nonspecific. x 40.

Fig. 15—Higher magnification of fig. 14. Loose mononuclear infiltration around a portion of the vascular circumference. The endothelium is not involved. x 337.

cephalomeningitic conditions may be discovered, such as a condition resembling lymphocytic choriomeningitis, which eventually may become established as definite entities.

STATISTICS**

In order to increase the magnitude of the sample, the data on 124 brains from the previous report[6] were combined with the present data on 155 brains and presented in table 1.

In attempting a statistical evaluation of the data, the question was whether or not hogs affected with hog cholera exhibit encephalitic lesions, regardless of their type and distribution, more frequently than nonaffected hogs. It can be seen from table 1 that animals which had hog cholera showed a high incidence of encephalitis. To eliminate any question of statistical significance, the 2 x 2 contingency tables of Fisher and Yates[3] were applied:

TABLE 1.—Incidence of Encephalitis Lesions in Porcine Brains

Type of material	Examined (No.)	Affected (No.)	Affected (%)
Hog cholera (HC)			
Field, uncompl.	65	61	95
Field, compl.	14	14	100
Transmission exper.	6	6	100
Hyperimmunized with virus	2	0	0
Simultaneously vacc.	15	0	0
Rovac vacc.	8	0	0
Rovac vacc. and challenged	10	1*	10
Nervous disorders other than HC			
Listeriosis	1	1	100
Lymphocytic choriomeningitis	2	2	100
Meningitis, purulent	1	1	100
Infectious diseases other than HC			
Erysipelas	3	0	0
Gastroenteritis	7	0	0
Moniliasis	1	0	0
Omphalitis	3	0	0
Pasteurellosis	6	0	0
Salmonellosis	9	0	0
Tetanus	2	0	0
Tuberculosis	1	1*	100
Noninfectious diseases			
Anemia	22	2*	10
B avitaminosis	19	0	0
Hypoglycemia	6	1*	16
Nephrosis	1	0	0
Poison (parathion, etc.)	13	1*	8
Unidentified deficiencies	6	0	0
Controls			
Nonvacc. for HC	22	0	0
Nonvacc. for HC (from Canada)	34	2*	6
Totals	279	93	33

*Indicates small lesion, not typical of hog cholera encephalitis.

**In this analysis, the authors wish to acknowledge the help of Dr. G. Beall, Department of Mathematics, University of Connecticut.

TABLE 2— 2 x 2 Contingency Table

	Hog cholera	Non-hog cholera	Total
Encephalitis	81	12	93
No encephalitis	4	182	186
Total	85	194	279

$$X^2_c = \frac{(12.5 \times 415 - 80.5 \times 181.5)^2 \times 279}{85 \times 194 \times 186 \times 93} = 207.19;\ P<0.001$$

Thus, the statistically highly significant incidence of encephalitic lesions and the incidence of hog cholera are interdependent.

STATISTICS**

For the purpose of evaluating the neuropathological diagnosis of hog cholera, an additional group of 155 porcine brains was examined. In this group, 35 represented hog cholera, 20 hog cholera-vaccinated by various methods, 4 definite neurological diseases and 8 other infectious diseases, 52 noninfectious diseases, and 36 controls, mostly from an hog cholera-free area (Canada).

All of the hog cholera cases showed typical nonpurulent disseminated encephalitis. In 5 cases, the diagnosis was confirmed by transmission experiments. Four cases represented definite neurological entities; namely, listeriosis, purulent meningitis complicating pleuritis, and a condition resembling lymphocytic choriomeningitis. Among 116 pigs either vaccinated or affected with various infectious and noninfectious diseases, and among the controls, only 4 specimens presented small isolated cellular foci, usually in the thalamus. The alterations were similar in vaccinated and challenged pigs, in cases of poisoning, and in Canadian controls and, therefore, were considered nonspecific.

For statistical evaluation, the previously reported and present data were combined and treated according to the method of 2 x 2 contingency tables; a P value of <0.001 was obtained. Expressed in another way, the incidence of all types of encephalitic lesions in the total 279 brains was 33 per cent, primarily owing to the inclusion of hog cholera cases; the incidence in 85 cases of the latter disease was 95 per cent.

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Some Properties of the Causative Agent of Transmissible Gastroenteritis in Swine

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SINCE TRANSMISSIBLE gastroenteritis was described by Doyle and Hutchings in 1946,[3] recognition of the economic importance of this disease has increased rapidly. It is now recognized as a major cause of baby pig losses in densely populated swin-producing areas. It has been reported in all of the Midwestern states of the United States, and in some, it has caused the death of many thousands of baby pigs. Due to the rapidity with which the disease spreads and kills young pigs, treatment and control up to this time have been very discouraging.

A review of the literature will not be included in this report, since it has been adequately presented in a previous report by Bay *et al.*[1]

EXPERIMENTAL STUDIES

The studies reported here were made for the purpose of clarifying some of the characterists of the causative agent and to observe the effect of treatment. The following phases were studied: (1) the survival of the causative agent under various conditions; (2) the effect of centrifugation on the concentration of the causative agent; (3) the susceptibility of laboratory animals to the causative agent; and (4) the effect of treatment. The results include data on 96 baby pigs, 19 guinea pigs, 43 mice, 4 hamsters, and 5 rabbits.

Pigs were removed from the farrowing pens when from 1 to 5 days old and placed in cages. The method of handling and feeding were the same as previously reported by Bay *et al.*

All of the mice, guinea pigs, hamsters, and rabbits used in this study were placed in sterilized metal cages on the day of exposure.

All tissues and gastro intestinal contents used in exposing pigs and laboratory animals were ground in a sterile Waring blendor. The finely divided material thus obtained was used either undiluted or diluted with sterile physiological salt solution to the desired concentration. Wherever Berkfeld N filtrates were used, the tissues were first ground and then centrifuged at 8,000 revolutions per minute (r.p.m.) for three minutes, then the supernatant fluid was filtered. Stock supplies of tissues and filtrates from affected pigs were placed in a freezer for storage at —28 C. Two cubic centimeters of the material were used in making exposures, unless otherwise specified.

RESULTS

SURVIVAL STUDIES

Putrefaction and Drying at 67 to 70 F.—Ten pigs, 1 to 5 days old, were exposed *per os* to ground gastrointestinal tract that had been left to putrefy and dry at 67 to 70 F. One of 2 pigs fed gastrointestinal tract after one day and 2 of 4 pigs fed gastrointestinal tract after three days of drying died of gastroenteritis. One of 2 pigs fed gastrointestinal tract after one day, 2 of 4 pigs fed gastrointestinal tract after three days, and 4 of 4 pigs fed gastrointestinal tract after ten days of drying lived.

Drying over Sulfuric Acid.—A total of 8, 1- to 5-day-old pigs were used to determine the effect of drying active gastrointestinal tract over sulfuric acid. Two pigs fed the dried gastrointestinal tract three days after the drying began died of gastroenteritis. However, 4 pigs fed ten days and 2 fed twenty-three days after the drying began all lived.

Formalin Sensitivity.—Fourteen pigs, 1 to 5 days old, were exposed *per os* to the supernatant fluid from centrifuged ground active gastrointestinal tract containing formalin. The mixture of formalin and supernatant fluid was incubated at 37 C. for twenty minutes prior to inoculation. One of 2 pigs fed the supernatant fluid containing 1 per cent formalin died. Normal pigs fed saline containing 1 per cent formalin also died. No lesions of transmissible gastroenteritis were seen in these pigs. One of 2 pigs fed supernatant fluid

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containing 1 per cent formalin and all pigs fed supernatant fluid containing .5 per cent, .25 per cent, .1 per cent, and .05 per cent formalin lived.

Storage at —28 C.—Previously, it was noted that active ground gastrointestinal tract and filtrates stored at —28 C. for a year regularly produced disease in 2- to 5-day-old pigs. To get further information as to the length of time the agent survives at —28 C., samples stored three years and six months, two years and five months, and one year were fed to pigs 2 to 5 days old. Six pigs were used in these trials. All 6 pigs died of gastroenteritis.

THE EFFECT OF CENTRIFUGATION ON THE CONCENTRATION OF THE CAUSATIVE AGENT

Filtrates from ground gastrointestinal tracts of infected pigs were centrifuged at 15,000 to 18,000 r.p.m. for varying lengths of time. The sediment and supernatant fluid were then separated and given to 10, 1- to 5-day-old pigs by way of a stomach tube. Table 1 shows the results.

All of the pigs fed the sediment from centrifuged filtrates died of gastroenteritis. There was no appreciable difference between the length of survival of the pigs fed the centrifuged filtrates and that of the pigs given uncentrifuged filtrates. One of 4 pigs fed the supernatant fluid died on the same day as the controls. The remaining 3 pigs fed the supernatant fluid lived two to four times as long as the control animals.

LABORATORY ANIMAL INOCULAION

Guinea Pigs.—Ten guinea pigs, 2 to 4 days old, and 9 adult guinea pigs were exposed *per os* or subcutaneously with active transmissible gastroenteritis filtrates or ground gastrointestinal tract. No evidence of infection was observed in any of these guinea pigs.

Mice.—Thirty-five mice, 1 day old, and 8 adult mice were exposed intraperitoneally, *per os*, or subcutaneously with active filtrates or ground gastrointestinal tract. None of the 43 mice used in these trials showed any evidence of disease.

Rabbits.—Five rabbits, 2 adults and 3 weanlings, were exposed to active gastroenteritis material. The 2 adults were exposed *per os* and intravenously. The 3 weanlings were injected intravenously. No symptoms of gastrotenteritis were seen in any of these rabbits. Tissues taken from these weanlings three and eight days following inoculation produced no disease when fed to young pigs.

Hamsters.—Four hamsters, 2 adults and 2 which were 3 weeks old, were exposed *per os* with ground gastrointestinal tract from infected pigs. None of the 4 hamsters developed any symptoms suggestive of gastroenteritis.

TABLE 1— Results of Exposing Pigs to Supernatant Fluid and Residue Resulting from Centrifugation

No. pigs	Exposure material	No. days lived after exposure	Length of life, controls (days)	Died gastroenteritis	Centrifuge time (minutes)
1	Sediment	2	3	1	30
3	Sediment	3	3	3	30
1	Sediment	3	3	1	60
1	Sediment	4	3	1	60
1	Supernatant	3	3	1	30
1	Supernatant	..	3	killed at 1 week	30
1	Supernatant	13	3	1	60
1	Supernatant	6	3	1	60

THERAPY

In Vitro.—*In vitro* studies using streptomycin, aureomycin, penicillin, and combinations of streptomycin and penicillin were conducted. A mixture of the antibiotic and causative agent was left at room temperature (68 to 70 F.) for one hour before being given to 2- to 5-day-old pigs. The results are shown in table 2.

It appears that the causative agent of transmissible gastroenteritis was not affected by any of the antibiotics used except possibly aureomycin.

Effects of Antibiotics In Vivo.—Various antibiotics have been used in an effort to find a treatment for transmissible gastroenteritis. A total of 32 pigs, 1 to 5 days

TABLE 2—Effect of Antibiotics In Vitro

No. pigs	Antibiotic	Amount antibiotic per cc.	Died of gastroenteritis	Remained well
2	Penicillin	2500 U./cc.	2	..
6	Aureomycin	12 mg./cc.	2	4
2	Streptomycin	50 mg./cc.	2	..
2	Streptomycin	33 mg./cc.	2	..
4	Streptomycin	25 mg./cc.	4	..
	Penicillin	2500 U./cc.	..	..

old, were used in these studies. Table 3 shows the results of this study.

None of the various treatments used had any noticeable effect on the course of the disease in artificially infected pigs. Only 1 pig in this group lived. It was treated with chloromycetin. This pig showed some diarrhea following exposure but recovered.

DISCUSSION

The results of the studies reported here indicate that the causative agent of transmissible gastroenteritis is rather short lived outside the animal body under natural conditions. It is very sensitive to low concentrations of formalin but remains active for an indefinite period when stored at —28 C.

By centrifuging filtrates for thirty minutes to one hour at 15,000 to 18,000 r.p.m., a relative centrifugal force of 20,000 to 25,000 x gravity, the causative agent was apparently concentrated to some extent in the sediment. Nevertheless, there were sufficient quantities of the causative agent in the supernatant fluid to reproduce the dis-

TABLE 3—Results of Treatment of Gastroenteritis in Baby Pigs

Kind of treatment	No. pigs	Dosage	Exposure material	Length treatment	Treatment started	Lived after exposure
Circulin orally	2	25 mg. daily	2 cc. ground G.I.* tract	2 days	day of exposure	2 & 3 days
Circulin orally	2	25 mg. daily	2 cc. ground G.I. tract	1 day	After onset of symptoms	3 & 3 days
Streptomycin intramuscularly	2	125 mg. twice daily	2 cc. ground G.I. tract	2 days	After onset of symptoms	3 & 4 days
Streptomycin intramuscularly	2	125 mg. twice daily	2 cc. ground G.I. tract	3 days	Day of exposure	3 & 4 days
Aureomycin orally	4	50 mg. twice daily	2 cc. ground G.I. tract	3 days or death	Day of exposure	2 & 3 days
Streptomycin intramuscularly Sulfadiazine orally	2	125 mg. twice daily 1 Gm. per lb. body weight	2 cc. ground G.I. tract	2 days	day of exposure	2 & 2 days
Streptomycin intramuscularly	2	150 mg. twice daily	2 cc. ground G.I. tract	2 days	Day of exposure	6 & 6 days
Streptomycin intramuscularly Sulfadiazine orally Aureomycin orally	2	125 mg. twice daily 1 Gm. per lb. body wt. 50 mg. twice daily	2 cc. ground G.I. tract	2 days	Day of exposure	2 & 2 days
Aureomycin orally Streptomycin intramuscularly	2	50 mg. twice daily 125 mg. twice daily	2 cc. ground G.I. tract	2 days	Day of exposure	2 & 4 days
Aureomycin orally Sulfadiazine orally	2	50 mg. twice daily 1 Gm. per lb. body wt.	2 cc. ground G.I. tract	2 days	Day of exposure	2 & 2 days
Streptomycin intramuscularly Sulfamethazine intraperitoneally	2	150 mg. twice daily 1 Gm. per lb. body wt.	2 cc. ground G.I. tract	2 days	Day of exposure	3 & 3 days
Streptomycin orally	2	500 mg. twice daily	2 cc. ground G.I. tract	2 days	Onset of sympoms	4 & 4 days
Streptomycin orally	2	500 mg. twice daily	2 cc. ground G.I. tract	4 days	Day before exposure	3 & 3 days
Sulfathaladine and arsenic orally	2	1 Gm. per lb. body wt.	2 cc. ground G.I. tract	.....	.....	.....
Chloromycetin orally	2	100 mg. twice daily	2 cc. ground G.I. tract	3 days	Onset of symptoms	5 and 1 taken off experiment

*Gastrointestinal.

ease. Centrifuging may be a means of concentrating the agent for further studies.

As yet, no laboratory animal has been infected with the causative agent. If the disease could be established in some laboratory animal, further research would be facilitated. At present, the only means of diagnosis are the symptoms and autopsy findings observed in infected pigs. Hence, the number of animals available for experimentation is limited and seasonal.

None of the antibiotics used was found to have merit in the treatment of gastroenteritis. The time between onset of symptoms and death in young pigs is usually short, hence an effective treatment would have to be quick-acting. If means were available for prevention, it would be of great value, since additional pigs are usually farrowed during or shortly after an outbreak of the disease.

SUMMARY

1) The causative agent of transmissible gastroenteritis remained infective for young pigs after three days' drying at 67 to 70 F.

2) Supernatant fluid from centrifuged ground gastrointestinal tract containing .05 per cent formalin was not infective.

3) Ground gastrointestinal tract produced disease after being stored for three years and six months at —28 C.

4) Centrifugation of filtrates at 15,000 to 18,000 revolutions per minute for thirty to sixty minutes apparently concentrated the causative agent to some extent.

5) No host except swine has been found for the causative agent of transmissible gastroenteritis.

6) Under the conditions of this study, none of the therapeutic agents used was of any value in the treatment of transmissible gastroenteritis.

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Studies on Baby Pig Mortality
X. Influence of Environmental Temperature on Fasting Newborn Pigs

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IN CONNECTION WITH an experiment designed to study the chemical and morphological aspects of hypoglycemia in fasted newborn pigs,[2, 3] it became evident that duration of life might be affected to a significant degree by the temperature of the environment. Accordingly, 26 pigs from seven litters were taken from the sows at farrowing, divided into two groups, and fasted: group 1 (11 pigs) at a mean environmental temperature of 59 F. (range 57 to 61 F.) and group 2 (15 pigs) at 88 F. (range 82 to 93 F.). The lower temperature was obtained by means of a large metal pail suspended in a larger metal tub, with cold running tap water constantly surrounding the inner vessel (fig. 1). By leaving the top of the chamber uncovered to permit ready dissipation of the body heat lost, the rather constant temperature was obtained. The warmer environment was produced by a 200-watt incandescent bulb covered by a metal hover in a cubicle (fig. 2) placed in a steam-heated room. Otherwise, the procedure was the same as used in the earlier experiment with the following exceptions. Body weight loss and weight of the liver at time of autopsy were determined by means of a Toledo direct-reading balance accurate to one Gm. Final body temperatures were determined rectally. The kidneys of 22 pigs (11 from

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TABLE I—Influence of Environmental Temperature Upon Fasted Pigs

	Group 1 (11 pigs)		Group 2 (15 pigs)	
	Mean	Range	Mean	Range
Environmental temperature (F)	59.0	57-61.0	88.0	82-93.0
Time until moribund (hr.)	28.0	20-33.0	84.0	61-124.0
Final body temperature (F.)	<94.0	<94.0	<96.4	<94-103.4
Body weight loss (%)	11.9	6.2-17.8	30.9	19.8-40.9
Final weight of liver (Gm.)	23.0	14-32.0	16.0	10-24.0
Chemical analyses on moribund pigs				
Whole blood				
Hemoglobin (Gm.%)	13.6	8.0-15.3	15.8	11.0-19.8
Glucose (mg.%)	12.9	6.8-24.9	11.6	6.8-22.6
Nonprotein nitrogen (mg.%)	121.4	82.0-156.9	175.8	70.6-273.5
Urea nitrogen (mg.%)	39.1	25.3-56.2	121.4	60.0-253.5
Creatinine (mg.%)	1.8	1.0-2.8	2.1	0.6-2.7
Uric acid (mg.%)	1.2	0.7-1.7	1.7	0.6-5.4
Chlorides (mg.%)	448.0	405-485.0	457.0	375-490.0
Serum				
Calcium (mg.%)	9.9	7.5-11.5	8.0(10)*	6.1-10.4
Inorganic phosphorus (mg.%)	10.1(8)*	8.0-11.5	8.8(8)*	7.2-10.8
Urine**				
Total nitrogen (mg.%)	880.7(5)*	623.3-1169.2	1554.3(6)*	1200.00-1831.2
Ammonia nitrogen (mg.%)	23.8(2)*	12.4-35.2	69.8(6)*	14.9-249.6
Uric acid (mg.%)	66.2(5)*	35.0-94.9	79.3(6)*	22.7-184.5
Tissue				
Liver glycogen	trace	0.0-trace	trace	0.0-trace
Kidney uric acid (mg./100 Gm.)	7.4	2.5-15.3	20.5	0.6-71.8

*No. of pigs on which data are available, if less than the total. **These values represent only random samples collected at time of euthanasia; therefore can not be considered strictly quantitative.

each group) were examined by means of a binocular dissecting microscope (at x 21) for the presence of urates. Experimental results are summarized in tables 1 and 2.

TABLE 2—Occurrence of Macroscopic Urates in Kidneys

	Group 1	Group 2
—	11	4
±	0	3
+	0	0
++	0	3
+++	0	1
++++	0	0

— = negative for macroscopic crystals; ± = up to four or five tubules on cut surface contain crystals; + to ++++ = varying amounts of urates from a few flakes in each papilla to an abundant orange precipitate in papillary ducts and pelvis.

DISCUSSION

Obviously, significant differences between the two groups appear in (1) time elapsing before they became moribund; (2) body weight loss; (3) blood nonprotein nitrogen levels, particularly that of urea nitrogen; and (4) concentration of uric acid in the kidneys. Other apparent differences which may have significance are those in (1) final weight of liver and (2) hemoglobin concentration. The significance of these latter differences can not be determined accurately, since (1) the effects of dehydration can not be measured and (2) the reason for the difference in liver weights is not known. It appears likely that dehydration, which was obviously much greater in the pigs held in the warmer environment, may have been responsible for much of the loss of body weight in both groups, and perhaps, for much of the difference in liver weights also.

Probably the most significant results, however, are those which indicate no significant variation between the two groups: *viz.*, blood glucose and liver glycogen levels and body temperatures. These indicate that the common denominator in the two groups was depletion of the carbohydrate reserves. It seems only logical then to assume that the almost complete exhaustion of carbohydrates was the most critical result of fasting, and that the critical stage was postponed by the higher enviromental temperature. The greater increase in levels of total blood nonprotein nitrogen, and certain of its component fractions, and of kidney uric acid in group 2 are probably explainable on the basis of increased protein catabolism and dehydration. Even so, the gluconeogenetic mechanism was ineffective in preventing critical hypoglycemia.

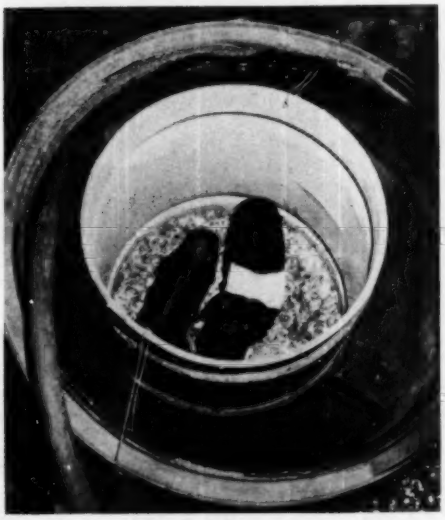

Fig. 1—A cool environment (57 to 61 F.) was obtained by surrounding a metal container with cold running tap water and leaving it uncovered to permit ready dissipation of body heat.

These data suggest that the warmer environment so reduced body heat loss and conserved energy stores that life was prolonged approximately 300 per cent. If this is a sound conclusion, the fact should have considerable practical significance. Brooders have been credited by Foster and Vestal[1] with saving an average of 1½ pigs per litter during February-March farrowing through prevention of chilling and being overlain. This experiment indicates that one important effect of chilling may be that of rapid depletion of available carbohydrates. The importance of environmental temperature would be even greater in the event of complete or even partial agalactia in the sow. Even though the temperature of the cooler environment in this experiment would be considered far from extreme, it should be remembered that the pigs were denied any benefit of the dam's body heat. Even in the event of healthy, vigorous

pigs and ample milk from the dam, it appears likely that pig livability and rate of gain would be greater in cold weather when artificial heat is provided. Additional data are needed on the latter point.

coneogenesis in the pigs which lived longer. Even so, critical hypoglycemia was not prevented under conditions of complete fasting, but only delayed. Through taking advantage of this delay, the use of pig

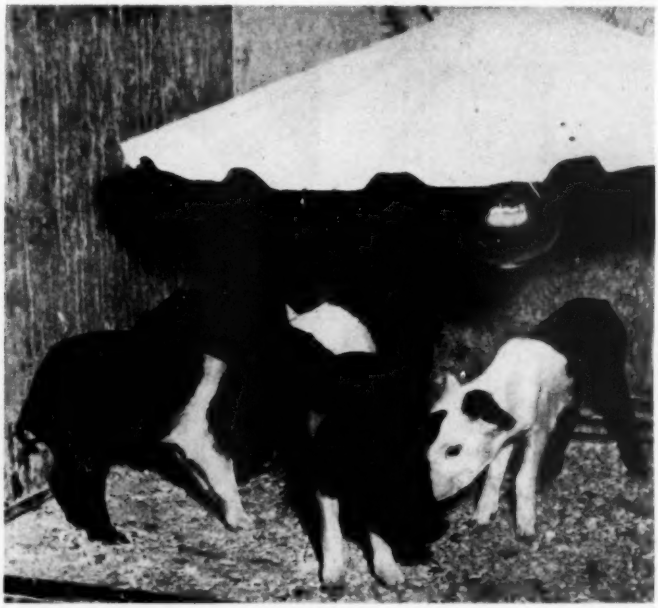

Fig. 2—A warm environment (82 to 93 F.) was maintained by means of a 200-watt incandescent bulb covered by a metal hover in a cubicle placed in a steam-heated room.

SUMMARY AND CONCLUSIONS

An experiment designed to study the influence of enviromental temperature upon fasting newborn pigs is described and the significance of the results discussed.

It was found that the rate of development of critical hypoglycemia varied inversely with the temperature of the environment. Higher temperatures evidently conserved the natal carbohydrate reserves through reduction in rate of heat loss. However, the data suggest that this effect was augmented to some extent by glu-

brooders in cold weather farrowing may save pigs whose dams have agalactia, provided the agalactia is incomplete or transient or is soon overcome by treatment.

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Studies on Baby Pig Mortality. XI. A Note on the Influence of Fasting on Body Temperature, Body Weight, and Liver Weight of the Newborn Pig

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GRAHAM, SAMPSON, AND HESTER[1] have reported that hypoglycemia in the newborn pig is likely to be accompanied in its later stages by subnormal body temperature. It has been observed also that fasting is accompanied by loss of body weight. In connection with a study of other aspects of the fasting syndrome, an attempt was made to obtain some measure of these changes. The purpose of this note is to report these observations.

EXPERIMENTAL

Body Temperature.—Data on two groups of newborn pigs are summarized in table 1. They were kept under similar conditions, except for the fact that one group was fasted until moribund and the other, as controls, were allowed to suckle.

Body temperatures were measured rectally with clinical thermometers on which the lowest graduation was 94 F. Only 4 of the fasted pigs registered above 94 and the highest reading was 95.8. Since most of them were well below 94 and some probably below 90, there is no doubt that the average was below 94. Eight of the fasted pigs had blood sugar levels so low that they were recorded only as a "trace." If these were computed as 0.0 mg. per 100 cc. of blood the mean would then be 8.7 mg.

TABLE 1—Effect of Fasting on Body Temperature in Newborn Pigs

		Fasted (22 pigs)		Suckled (17 pigs)	
		mean	range	mean	range
Time	hr.	49.0	24-80.0	64.7	26 - 80.0
Body temperatures	F.	<94.0	<94-95.8	102.4	101.1-103.4
Blood sugar levels	mg.%	<13.6	tr.*-47.5	115.8	79.1-135.6

*tr. = trace: insufficient to measure.

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Body Weight.—Initial and final weights in grams are available on two groups of newborn pigs: 58 which were fasted until moribund and 26 which were allowed to remain with the sows. Weights were obtained by means of a Toledo direct-reading balance accurate to 1 Gm. The data on body weights are summarized in table 2 and the averages pictured graphically in fig. 1.

Liver Weights.—When the experiment was partially completed, an impression was formed that the livers of fasted and suckled pigs might be disproportionate in size. Accordingly, weights to the nearest gram were obtained on the fresh livers of 33 fasted pigs and 12 suckled pigs. These weights are summarized and compared with final body weights in table 3.

From these data, it would appear that the liver loses considerably more weight than the average for body tissues of newborn pigs during fasting. It might be expected since the

FIGURE 1

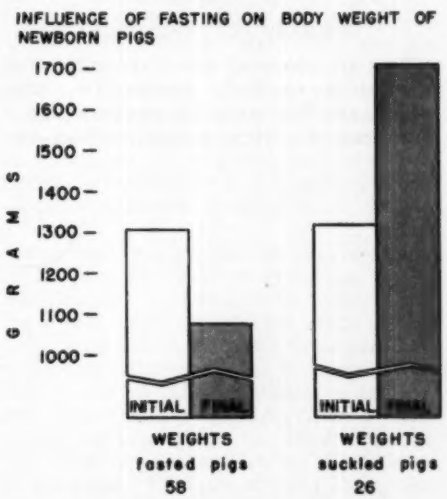

Fig. 1—Influence of fasting on body weight of newborn pig.

hepatic glycogen store is exhausted during that time. However, this is inadequate to explain the disparity between the two groups, since the author[2] has shown that the hepatic glycogen in pigs similar to these suckled pigs contained, on an average, only 2.7 per cent by weight on a fresh basis. The rest of the difference must be explained on the basis of other phenomena such as dehydration.

TABLE 2—Effect of Fasting on Body Weight in Newborn Pigs

		Fasted (58 pigs)		Suckled (26 pigs)	
		mean	range	mean	range
Time	hr.	49.4	4-120.0	67.1	26-156.0
Initial weight	Gm.	1303.7	811-1875.0	1319.6	928-1724.0
Final weight	Gm.	1076.5	633-1498.0	1707.3	1190-2524.0
Gain	%	.....	.....	30.5	.3-101.6
Loss	%	17.3	2.1-37.0	.....	.....

TABLE 3—Influence of Fasting on Fresh Weight of Liver in Newborn Pigs

		Fasted (33 pigs)		Suckled (12 pigs)	
		mean	range	mean	range
Time	hr.	46.10	10-105.00	59.10	26-144.00
Final liver weight	Gm.	19.60	13-27.00	46.40	35-63.00
Final body weight	Gm.	1035.30	633-1398.00	1541.50	1190-1792.0
Liver : body weight	ratio	1 : 52.80	1 : 40.9-1 : 64	1 : 33.20	1 : 23.8-1 : 42.10
Liver : body weight	%	1.89	1.56-2.44	3.01	2.38-4.20

SUMMARY AND CONCLUSIONS

Data are presented which show the effect of fasting on body temperature, body weight, and liver weight in newborn pigs.

Fasting of newborn pigs, accompanied by hypoglycemia, results in its terminal stages, at least, in marked hypothermia, loss of body weight, and loss in liver weight. In this experiment, terminal body temperatures were generally below 94 F., average body weight loss was 17.3 per cent, and liver weight loss apparently was still greater.

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Studies on Baby Pig Mortality.
XII. A Note on the Influence of Ingestion of Distilled Water, Physiological Saline, and Glucose Solutions on Fasting Newborn Pigs

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EARLIER REPORTS[1, 2] from the Illinois Agricultural Experiment Station have shown that complete fasting of newborn pigs produces critical hypoglycemia in a relatively short time, and that the time required varies with the environmental temperature.[3] In connection with the latter observation, it was noted that body weight loss in 15 pigs held at a mean environmental temperature of 88 F. averaged 30.9 per cent, while in 11 pigs held at a mean temperature of 59 F., the average weight loss was 11.9 per cent. The time elapsing before the critical or terminal stage was reached averaged eighty-four hours for pigs kept in the warmer environment and twenty-eight hours for those kept at the lower temperature.

In view of so great a weight loss in so short a time, the question arose as to the relative importance of hypoglycemia, dehydration or, possibly, loss of electrolytes. In other words, was the condition of the fasted pig *in extremis* due chiefly to hypoglycemia or were other factors such as dehydration and disturbance of salt balance of as great relative importance under these conditions? In an effort to obtain a tentative appraisal of this situation, 48 pigs divided into four groups of 15, 11, 11, and 11, respectively, were treated as follows.

MATERIALS AND METHODS

All pigs were fasted at a mean environmental temperature of 88 F. (range 82-93 F.) maintained in the manner described earlier.[3] Group 1 (15 pigs) was fasted completely, receiving no fluids; group 2 (11 pigs) received distilled water; group 3 (11 pigs) received physiological saline solution; and group 4 (11 pigs) received 50 per cent glucose solution. All fluids were given *ad libitum* by mouth and at the same intervals. Clinical, postmortem, and chemical examinations were carried out in the manner described in an earlier report.[3] In addition, analyses for blood-free phenols were conducted in an effort to determine the presence of nitrogenous blood fractions which might be toxic. The method of Theis and Benedict, as cited by Hawk and Bergeim,[4] was used. The results of this experiment are summarized in table 1.

The appearance of macroscopic urate crystals in the kidneys is summarized in table 2.

DISCUSSION

Amounts of fluids consumed were relatively small, possibly because the pigs first had to learn to drink from a shallow dish. Ingestion of fluids tended to lessen body weight loss, although that effect was not proportionate to the amount of fluid ingested. The fluids also appeared to assist in temperature regulation, but only the glucose solution was effective in preventing hypothermia. Only glucose solution prevented development of hypoglycemia and virtually complete exhaustion of hepatic glycogen.

Azotemia was greatest in pigs receiving no fluids, least in those receiving glucose solution, and intermediate in those receiving water and saline. This would appear to be a logical result, since those receiving water and saline should be in a better position to excrete excess nitrogenous components, while those receiving glucose solution should, because of its protein-sparing action, show in addition a slower development of azotemia. Evidence of the diluting and flushing action of the fluids is seen in the absence or lesser concentrations of macro-

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scopic urate crystals in the kidneys of animals receiving them; results of chemical analysis of kidneys for uric acid also support such a conclusion.

It is interesting to note that, although body weight loss was as great in the pigs receiving glucose as in those receiving saline solution, the weight of the liver averaged 50 per cent greater. This would not appear to be accounted for solely on the basis of the hepatic glycogen which averaged 3.8 per cent (fresh weight).

(Morrill) previously reported calcium levels of 7.5 to 14.5 (ave. 12.0) mg. per 100 ml. of blood serum in 33 normal pigs, and inorganic phosphorus levels of 5.88 to 8.80 (ave. 7.4) mg. per 100 ml. of blood serum in 27 normal pigs of comparable age.[2] At the same time, it was reported that 39 pigs fasted until moribund had calcium levels of 7.5 to 14.5 mg. per 100 ml. of blood serum but averaged 10.3, and 18 fasted pigs had inorganic phosphorus levels of 5.9 to 11.5 mg. per 100 ml. of blood serum and aver-

TABLE 1—Influence of Ingestion of Certain Fluids on Fasting Newborn Pigs

		Group 1			Group 2			Group 3			Group 4		
		No Fluids			Distilled Water			Physiological saline			50% Dextrose solution		
		Mean	Range	No.*	Mean	Range	No.*	Mean	Range	No.*	Mean	Range	No.*
Time until destroyed	hrs.	84.0	61-124.0	15	70.0	51-118.0	11	72.0	57-80.0	11	70.0	45-85.0	11
Fluids consumed	ml.	0.0	0-0.0	15	132.0	64-301.0	11	245.0	105-430.0	11	214.0	108-418.0	11
Final body temperature	F.	96.4	94-103.4	15	97.9	95.4-100.4	10	97.9	94.6-101.0	10	102.2	97.8-104.6	11
Body weight loss	%	30.9	19.8-40.9	15	19.6	9.1-28.9	11	13.3	10.1-17.1	11	14.9	3.7-25.4	11
Final weight of liver	Gm.	16.0	10-24.0	13	15.0	10-21.0	11	15.0	10-23.0	11	22.0	16-30.0	11
Whole blood analyses													
Hemoglobin	Gm.%	15.8	11.0-19.8	15	13.2	10.0-16.0	11	12.1	8.7-14.0	11	11.7	6.6-15.0	11
Glucose	mg.%	11.6	6.8-22.6	15	12.2	0.0-27.1	11	13.3	2.3-27.1	11	133.2	52.0-254.2	11
Nonprotein nitrogen	mg.%	175.8	70.6-273.5	15	115.3	75.0-168.9	11	118.0	72.7-247.6	11	78.5	52.9-117.1	11
Urea nitrogen	mg.%	121.4	60.0-253.5	15	61.8	25.0-130.5	11	77.3	37.5-129.9	11	34.1	16.3-70.8	11
Creatinine	mg.%	2.1	0.6-2.7	15	2.4	1.9-3.5	11	2.0	1.3-2.9	11	2.0	1.3-2.8	11
Uric acid	mg.%	1.7	0.6-5.4	15	0.9	0.1-3.1	11	0.9	0.3-1.8	11	0.8	0.1-1.9	11
Chlorides	mg.%	457.0	375-490.0	15	449.0	400-487.0	11	445.0	380-485.0	11	438.0	370-490.0	11
Free phenols	mg.%	2.9	1.4-5.0	6	1.9	1.5-2.3	11	1.8	1.2-3.2	11	1.5	0.5-3.9	11
Serum analyses													
Calcium	mg.%	8.0	6.1-10.4	10	10.4	9.0-13.0	9	10.8	10.0-12.5	7	11.4	9.0-15.0	8
Inorganic phosphorus	mg.%	8.8	7.2-10.8	8	9.5	9.2-11.2	4	8.9	7.7-9.5	6	8.0	5.9-9.6	8
Urine analyses**													
Total nitrogen	mg.%	1554.3	1200-1831.0	6	1294.5	1171-1454.0	5	1328.6	980-1536.0	4	1111.4	792-1371.0	4
Ammonia nitrogen	mg.%	69.8	14.9-249.6	6	52.6	20.0-92.3	5	51.4	35.6-75.5	4	73.3	60.2-87.9	4
Uric acid	mg.%	79.3	22.7-184.5	6	109.8	53.8-156.8	5	95.8	43.5-208.5	4	158.5	61.1-229.4	4
Tissue analyses													
Liver glycogen	%	tr.***	tr.-tr.	15	tr.	tr.-tr.	10	tr.	tr.-tr.	11	3.8	0.1-9.1	11
Kidney uric acid	mg./100 Gm.	20.5	0.6-71.8	15	9.7	1.1-33.8	11	4.1	1.2-9.2	10	6.9	2.5-15.5	10

*No. animals on which data are included. **Based on random samples only, collected postmortem. ***tr. = trace only, insufficient to measure accurately.

The pigs receiving glucose were destroyed at a time comparable to those receiving other fluids—not according to their physical or physiological state. Most of them would have lived somewhat longer; in fact, 4 of the 11 were still very vigorous and showed evidence of good appetite when killed. Some showed more weakness accompanying the loss of body weight. A few pigs in this group showed some gastritis at autopsy. It is believed that the concentration or marked hypertonicity of the glucose solution may have been responsible.

The results of urine analysis for nitrogenous wastes are not considered significant because of the small numbers and the fact that 24-hour samples were not available.

It will be noted that the calcium : phosphorus ratio of the serum was maintained more nearly normal in the pigs receiving glucose than in any other group. One of us

aged 9.0 mg. Also, in additional recent studies,[5] it has been found that fasting apparently results in an increased level of serum inorganic phosphorus in older pigs. A moderate decrease in calcium levels ap-

TABLE 2—Appearance of Urate Crystals in Kidneys of Newborn Pigs Fasted Completely and Those Receiving Certain Fluids

	Group 1* No fluids	Group 2 Dist. water	Group 3 Phys. saline	Group 4 Glucose
—	4	6	9	7
±	3	1	2	2
+	0	3	0	2
++	3	1	0	0
+++	1	0	0	0
++++	0	0	0	0

— = negative for macroscopic crystals (on frontal section). ± = up to four or five tubules show crystals. + to ++++ = varying amounts of urates from a few flakes in each papilla to an abundant orange precipitate in papillary ducts and pelvis. *data on only 11 pigs available.

pears to have occurred in this experiment, whereas Sampson *et al.*[5] reported no significant change in older pigs. Since fluctuations in calcium and phosphorus levels are known to occur in normal animals, care must be exercised in drawing conclusions. It is notable, however, that critical hypoglycemia failed to develop in their pigs, and that pigs given glucose solution in this experiment also maintained normal levels and ratios of calcium : phosphorus.

SUMMARY AND CONCLUSIONS

The effects of ingestion of certain fluids by fasting newborn pigs are reported. Fifteen pigs were allowed no fluids, 11 were allowed distilled water, 11 physiological salt solution, and 11 glucose solution in 50 per cent concentration. Effects upon body weight, body temperature, hepatic glycogen and kidney uric acid levels, and blood sugar and nonprotein-nitrogenous factors are cited in particular.

Within the limits of this experiment, ingestion of distilled water and physiological saline by fasting newborn pigs failed to prevent or delay development of critical hypoglycemia, although there was some evidence that it may have deterred dehydration, promoted renal excretion of nitrogenous wastes, and facilitated temperature regulation to some degree.

On the other hand, within the same limits, ingestion of glucose solution was effective in preventing critical hypoglycemia, hypothermia, and exhaustion of hepatic glycogen.

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Blood Chemistry of Equidae—Some Variations in Inorganic Phosphorus, Alkaline Phosphatase Activity, Calcium, and Magnesium in Blood Serum of Horses

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BECAUSE OF THE importance of good skeletal development to the usefulness of the horse, information contributing to the early detection and diagnosis of various kinds of skeletal unsoundness, or of conditions predisposing thereto, is of potential value in the production and maintenance of sound animals. Considerable attention has been given in recent years to theories that relate the occurrence of the more common kinds of skeletal disorders to metabolite deficiencies resulting from errors in diet. Efforts have been made to establish blood analyses as clinical procedures for the detection of such deficiencies and as aids in the diagnosis of disturbances in bone metabolism.

Among those factors in blood that are of recognized significance in relation to the adequacy of the diet for the development of structurally sound bone, phosphorus and calcium have been most extensively investigated. The literature on variations in serum phosphorus and calcium levels in the horse was reviewed in 1941 by Craige and Gadd.[1] They pointed out that recognition of changes due to dietary deficiencies or to other pathological conditions was often impossible because of the wide range of normal variations in healthy and apparently well-fed horses.

They presented for the Thoroughbred horse, a considerable body of data which demonstrated in normal animals of this breed a range of 2.0 to 8.0 mg. of inorganic phosphorus per 100 ml. of serum. The most important factor influencing variations within this range was age, season having a secondary influence. Total serum calcium in their normal animals ranged from 11.0 to 13.5 mg. per 100 ml. of serum, variations within the range being attributed to advanced pregnancy and to differences in season and in feeding. In view of the difficulties of interpreting the usual data on levels of serum phosphorus and calcium, Craige and Gadd proposed, as an aid in identifying primary disturbances in the blood phosphorus and calcium, the use of an empirical formula which coördinated the results of analyses of blood serum for inorganic phosphorus, total calcium, and protein. The authors attached greater significance to deviations from the "normal" value obtained from the application of this formula than to deviations from the normal mean or range for phosphorus and calcium considered either singly or together.

Much less study has been given to the significance of variations in the level of magnesium in the blood of the horse. Although serum magnesium has been thought not to vary widely in most domestic species under ordinary conditions, it was reported to be reduced in rickets and in certain types of grass tetany in cattle,[2] in the low-magnesium tetany of milk-fed calves,[3] and in equine transit tetany.[4] Millen and Eveleth[5] found it also lowered in equine encephalomyelitis but increased in moldy corn poisoning of horses. There appears, however, to be considerable difference in the normal ranges reported by different investigators. Eveleth[6] found in 10 horses a range of 2.00 to 3.00 mg. per 100 ml. of serum, Green *et al.*[4] found in 19 normal horses a range of 1.89 to 2.20 mg., whereas, in horses with equine transit tetany, the average level was reduced to 1.16 mg. Blunn, Howell, and Caldwell[7] reported a mean value of 2.7 mg. per 100 ml. of serum from 68 analyses on normal animals.

The level of alkaline phosphatase in blood serum is influenced by the osteogenic activity of the organism, not only in its relation to the laying down of new healthy bone in the course of natural growth, but also in its relation to the healing of fractures and to abnormalities of bone development such as occur in malignancies, rickets, scurvy, and hypothyroiditis.[8] The normal serum level in healthy animals seems to be characteristic of the species and varies with age, physiological condition and, to some extent, with the diet. In human beings, the determination of serum alkaline phosphatase is used as a reliable and sensitive criterion of the severity of rickets and as an indication of the rate of healing. However, in the horse, there has apparently been little application of this type of analysis. In a study of the changes in inorganic phosphorus and phosphatase activity in 12 foals from birth to 6 months of age, Earle (in press) observed a mean phosphatase activity of 109 Bodansky units per 100 ml. of serum at birth, a level

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which declined rapidly during the first forty-eight hours and then progressively more slowly to a mean of 16 Bodansky units at 6 months of age.

The present report presents results of analyses of more than 300 samples of blood serum from 182 horses. The data are offered as being of potential value in nutritional evaluation or clinical diagnosis in so far as they indicate the influence of some physiological and environmental factors on the levels of serum inorganic phosphorus, alkaline phosphatase activity, calcium, and magnesium in apparently healthy horses.

MATERIALS AND METHODS

The data assembled here were obtained from animals at the U. S. Department of Agriculture field stations at three different geographic locations: Miles City, Mont.; Middlebury, Vt.; and Beltsville, Md.

At the Montana Station, samples were obtained in April from 98 animals, some of which were of draft type and some of saddle type, ranging in age from 1 to 17 years. Pregnant or lactating mares were excluded. Some of these 98 animals had been wintered on the range without any supplementary feed. Others had been stable-fed throughout the previous winter on native hay and grain.

At the Vermont Station, samples were obtained from the stud of purebred Morgan horses, varying in number at different seasons from 35 to 51 and including all ages, at four different times during one year. These times were as follows: in October near the end of the grazing season; in January after two months or more of grain and hay feeding; in April near the beginning of the pasture season; and lastly, in August while pasture grasses were still abundant. A band of 17 pregnant or lactating mares was included in the last three of these samplings.

At the Maryland Station, samples of blood from a group of horses consisting of 4 Morgans, 1 Thoroughbred, and 14 draft animals were taken in November and again the following March. This group included no pregnant or lactating mares. These animals ranged in age from 4 to 20 years, and had been maintained throughout the year on oats and timothy or light-mixed-timothy hay without access to pasture.

Although the actual sampling was done at the different stations, all analyses were carried out in the laboratories at Beltsville. All blood samples were drawn under comparable conditions, *i.e.*, in the morning before the animals were fed and with as little excitement as possible. Animals from the pasture or range were kept confined in stables or corrals without feed during the previous night. The blood samples were treated as nearly alike as the prevailing conditions permitted. About 40 ml. of blood were drawn from a jugular vein, the serum removed from the cells, and the trichloroacetic acid filtrates for the determination of inorganic phosphorus made on the same day the blood was drawn. Phosphatase determinations were made only on samples obtained at Beltsville and Middlebury, where incubation of serum samples could be started within a few hours after the blood was drawn. The trichloroacetic acid filtrates, together with the serum samples for calcium and magnesium analyses, were shipped by air express to the laboratory at Beltsville, where the actual analyses were carried out.

Bodansky's procedures[9,10] were used for the determination of serum inorganic phosphorus and serum alkaline phosphatase. Serum calcium was determined by the usual Kramer and Tisdall method employing the Clark-Collip[11] washing technique. Serum magnesium was precipitated according to the procedure described by Godden,[12] the phosphorus in the precipitate being determined by the same method employed for inorganic phosphorus.

RESULTS

Montana Horses. — Data obtained on horses in Montana are summarized in table 1. The animals have been grouped according to age and system of feeding. When there were more than 11 animals in a group (as in groups 1, 2, and 3), a further division according to breed or type has been made.

Differences due to breed or type appear only among the yearlings (group 1). Here, the saddle-type animals show slightly higher mean levels of both inorganic phosphorus and magnesium. Further, regarding the serum phosphorus in this age group, the saddle yearlings also show a wider range or variation than do the draft yearlings. This wider range among the saddle animals appears also in groups 2 and 3, for which the mean levels for the two types of animals are about the same.

Variations due to age are most marked in inorganic phosphorus, which decreases with increasing age regardless of differences in nutrition. Significant changes in calcium and magnesium, which can be attributed to age, are not apparent. However, the effects of the differences in nutrition which prevailed could well mask small differences due to age.

Differences due to nutrition are demonstrated by the data from groups 3 and 4, which include the same age range but which

differed in the system of feeding. Group 3 was wintered on the range without any supplementary feed, whereas group 4 was wintered in the feedlot or stable on grain and native hay. The range-fed animals appear to differ from the stable-fed animals chiefly in having a slightly lower mean level of serium calcium, *viz.*, 11.50 ± .13 mg. per 100 ml. of serum as against 12.09 ± .32 mg. for the stable-fed group.

Vermont Horses.—The results of all analyses made on the Morgan horses at Middlebury, Vt., exclusive of the brood mares which are treated separately, are summarized in table 2. The animals have been grouped according to age, without regard to some slight dietary variations within the age groups. The groups varied in composition in successive samplings only as individuals were eliminated due to sale or accident. Analyses for magnesium were made only in April and August.

In general, these animals were maintained largely on pasture during the pasture season and on grain and locally produced hay during the winter. Exceptions were: the stallions (2 in group 3 and 1 each in groups 4 and 6), which were entirely stall-fed throughout the year; the young mares in groups 4 and 5, which were allowed only intermittent grazing; and the young animals in group 2. These last were with their dams on pasture prior to the first sampling in October. After weaning in the fall, they were fed a diet of mixed grain, alfalfa hay, and timothy hay. Of the 6 individuals which were carried through all samplings, 5 were kept on this diet throughout the year while 2 were allowed to graze during the summer preceding the August sampling.

With respect to the quality of the pastures, attention is called to the fact that a phosphorus deficiency in the soil of this area had been recognized. It was customary to treat all pastures periodically with a top dressing of superphosphate. However, at the time of the first sampling in October, it had been three and a half years since the last pasture treatment. In June, following the taking of the April samples, all pastures were again heavily treated with superphosphate.

Differences due to age are to be observed in both serum inorganic phosphorus and phosphatase activity. The mean level of inorganic phosphorus for all seasons decreased from 5.72 ± .12 mg. per 100 ml. of serum in the 6- to 16-month-old group to 2.67 ± .09 mg. in the 18- to 27-year-old group. The mean phosphatase activity for all seasons decreased from 13.4 ± .4 units per 100 ml. in the younger group to 6.1 ± .5 units in the aged group. A slightly lower mean level of magnesium appears in young animals than in those 2 years or more of age.

The entire group of Vermont horses sampled varied from season to season in the proportion of young animals included. Hence, in view of the marked influence of age on the levels of inorganic phosphorus and phosphatase activity, mean values for these two factors have not been calculated for the group as a whole for individual seasons.

Variations with season are apparent in serum inorganic phosphorus, phosphatase activity, and calcium. In all groups except the very young and the aged, inorganic phosphorus tended to be lowest in October at the end of the grazing season and to increase in January after a period of grain and hay feeding to mean levels which remained relatively unchanged in April, but which were still higher in August after two months of grazing pastures which had been freshly treated with superphosphate.

The phosphatase activity, on the other hand, was lowest during the winter while the animals were being fed on grain and hay and was highest during August after a summer of plentiful pasture and sunshine. The mean calcium level tended to be lower in April and August than in October and January.

Variations due to pregnancy and lactation are shown by the data obtained on 17 pregnant mares in January, April, and August (table 3). These mares ranged in age from 5 to 18 years, with a mean age of 11 years. For comparison, the mean values from a control group of 8 nonpregnant, nonlactating mares of like age, which were kept under the same nutritional and environmental conditions, are included in the same table.

The pregnant mares delivered foals in April, some before and some following the taking of the April blood samples.

The most noteworthy variations in the pregnant or lactating mares are: the drop in inorganic phosphorus about the time of parturition, the rise in phosphatase level

TABLE 1—Variations in Inorganic Phosphorus, Calcium, and Magnesium in Serum of Montana Horses in One Season (April)

Group (No.)	Age (Yr.)	Type	Feeding	No. of animals	Inorganic phosphorus (mg./100 ml. serum)		Calcium (mg./100 ml. serum)		Magnesium (mg/100 ml. serum)	
					Mean	S.E.‡	Mean	S.E.	Mean	S.E.
1	1	Draft*	Hay and grain	13	5.20	± .08	12.18	± .03	1.81	± .04
		Saddle**		17	5.77	± .15	12.10	± .08	2.02	± .04
		Total		30	5.50	± .09	12.13	± .03	1.93	± .03
2	2	Draft	Range grazing	8	5.34	± .15	11.96	± .13	2.13	± .08
		Saddle		19	5.25	± .24	11.70	± .09	2.12	± .03
		Total		27	5.28	± .15	11.78	± .07	2.12	± .04
3	3 to 8	Draft	Range grazing	10	4.23	± .13	11.36	± .21	2.09	± .07
		Saddle		10	4.20	± .25	11.65	± .17	2.09	± .04
		Total		20	4.21	± .14	11.50	± .13	2.09	± .04
4	3 to 8	Draft and saddle†	Hay and grain	10	3.96	± .21	12.09	± .32	1.91	± .06
5	9 to 17	Draft and saddle†	Range grazing	11	3.42	± .15	11.98	± .25	2.11	± .07
		Mean for total		98	4.80	± .05	11.89	± .06	2.03	± .02
		Range		....	2.97 to 6.33		10.73 to 13.11		1.61 to 2.37	

*Draft-type animals consisted of Belgians and grade Belgians. **Saddle-type animals consisted of Nonius, Thoroughbreds, Morgans, and crosses of these breeds. †Because of small numbers, both types have been treated together. ‡Standard error.

TABLE 2—Variations in Serum of Morgan Horses at Middlebury, Vt., in Four Successive Seasons

Group (No.)	Age	October			January			April			August		
		No. of animals	Mean	S.E.*	No. of animals	Mean	S.E.	No. of animals	Mean	S.E.	No. of animals	Mean	S.E.
Inorganic phosphorus mg. per 100 ml. serum													
1	4 to 5 mo.	....	.....		....	.....		....	.....		5	6.87	± .15
2	6 to 16 mo.	16	5.24	± .18	9	6.17	± .23	6	6.01	± .43	6	6.04	± .33
3	18 to 28 mo.	6	4.14	± .24	4	5.31	± .30	4	4.63	± .20	4	5.46	± .54
4	30 to 40 mo.	4	3.95	± .11	4	4.15	± .25	4	4.04	± .10	4	4.88	± .55
5	42 to 52 mo.	3	3.74	± .14	3	4.07	± .30	3	4.30	± .18	3	4.36	± .07
6	18 to 27 yr.	6	2.64	± .05	5	2.62	± .15	4	2.63	± .21	4	2.80	± .34
		Mean of 107 analyses = 4.53 ± .06; range = 2.18 to 7.48											
Alkaline phosphatase activity: Bodansky units per 100 ml. serum													
1	4 to 5 mo.	....	.....		....	.....		....	.....		5	16.2	± .7
2	6 to 16 mo.	16	13.4	± .5	9	12.2	± .7	6	14.9	± 1.3	6	14.2	±1.4
3	18 to 28 mo.	6	11.6	± .8	4	6.3	± .1	4	9.2	± .5	4	10.9	± .1
4	30 to 40 mo.	4	10.8	±1.4	4	5.4	± .4	4	6.4	± .5	4	7.3	±1.3
5	42 to 52 mo.	3	8.5	± .5	3	6.3	± .2	3	7.0	± .5	3	7.9	± .6
6	18 to 27 yr.	6	7.3	± .9	5	5.1	±1.2	4	5.2	± .4	4	6.6	± .4
		Mean of 107 analyses = 10.0 ± .2; range = 3.2 to 18.5											
Calcium: mg. per 100 ml. serum													
1	4 to 5 mo.	....	.....		....	.....		....	.....		5	12.06	± .05
2	6 to 16 mo.	16	11.85	± .22	9	12.19	± .27	6	11.90	± .12	6	11.85	± .19
3	18 to 28 mo.	6	12.08	± .38	4	12.61	± .04	4	11.40	± .87	4	11.80	± .43
4	30 to 40 mo.	4	12.80	± .17	4	12.42	± .15	4	11.82	± .27	4	11.23	± .05
5	42 to 52 mo.	3	12.79	± .16	3	12.06	± .14	3	11.45	± .42	3	11.68	± .41
6	18 to 27 yr.	6	12.76	± .34	5	12.29	± .14	4	12.17	± .45	4	13.54	± .33
Total 4 mo. to 27 yr.		35	12.21	± .10	25	12.30	± .11	21	11.78	± .19	26	11.88	± .12
Range		....	9.97 to 12.96		....	10.26 to 12.92		....	9.97 to 13.42		....	10.73 to 13.22	
		Mean of 107 analyses = 12.07 ± .07; range = 9.97 to 13.42											
Magnesium: mg. per 100 ml. serum													
1	4 to 5 mo.	....	.....		....	.....		....	.....		5	1.87	± .05
2	6 to 16 mo.	....	.....		....	.....		6	1.53	± .17	6	1.76	± .06
3	18 to 28 mo.	....	.....		....	.....		4	2.31	± .22	4	1.94	± .09
4	30 to 40 mo.	....	.....		....	.....		4	2.03	± .16	4	1.92	± .03
5	42 to 52 mo.	....	.....		....	.....		3	1.83	± .06	3	2.07	± .05
6	18 to 27 yr.	....	.....		....	.....		4	2.29	± .15	4	1.97	± .11
Total 4 mo. to 27 yr.		....	.....		....	.....		21	1.96	± .10	26	1.91	± .03
Range		....	.....		....	.....		....	1.25 to 2.65		....	1.59 to 2.24	
		Mean of 47 analyses = 1.93 ± .05; range = 1.25 to 2.65											

*Standard error.

near the time of parturition, the increase in phosphatase activity with lactation, and the marked drop in calcium with advanced lactation.

Maryland Horses.—Results of analyses made on 19 horses at Beltsville, Md., in November and again the following March are presented in table 4. None of these animals had access to pasture either during the summer preceding the first sampling or during the interval between samplings. Data have been treated according to three age groups, *i.e.*, 4 to 5, 9 to 11, and 16 to 20 years. Variations with age are apparent in the decreasing levels of inorganic phosphorus and phosphatase activity with increasing age. A variation with season is suggested only in the lowering of phosphatase activity in March, which occurred with each age group.

TABLE 3—Variations in Serum of Morgan Mares Due to Pregnancy and Lactation

Group (No.*)	Age	No. of animals	January Mean	January S.E.**	April Mean	April S.E.	August Mean	August S.E.
			Inorganic phosphorus: mg. per 100 ml. serum					
1	5 to 18 yr.	17	3.65	± .12	2.99	± .07	3.70	± .15
2	4 to 22 yr.	8	3.31	± .29	3.39	± .31	3.39	± .36
			Alkaline phosphatase activity: Bodansky units per 100 ml. serum					
1	5 to 18 yr.	17	5.5	± .43	8.00	± .46	9.70	± .65
2	4 to 22 yr.	8	5.2	± .51	6.00	± .38	7.20	± .41
			Calcium: mg. per 100 ml. serum					
1	5 to 18 yr.	17	12.09	± .10	11.42	± .11	10.35	± .29
2	4 to 22 yr.	8	12.46	± .14	11.98	± .35	12.00	± .26
			Magnesium: mg. per 100 ml. serum					
1	5 to 18 yr.	17	.....		1.97	± .08	1.89	± .06
2	4 to 22 yr.	8	.....		1.99	± .07	2.02	± .05

*Group 1 consists of 17 mares which delivered foals in April. Group 2 consists of 8 open mares on same pasture.
**Standard error.

Comparison of Data From Three Localities.—The mean values for serum inorganic phosphorus and phosphatase activity obtained from the various age groups of animals at the three geographic locations are plotted against age in fig. 1. The curves resulting indicate that geographic location has relatively little influence on these factors as compared with the influence of age.

Since age appears to have little influence

TABLE 4—Variations in Inorganic Phosphorus, Phosphatase Activity, and Calcium in Serum of Horses at Beltsville, Md., in Two Successive Seasons

Group (No.)	Age	No. of animals	November Mean	November S.E.*	March Mean	March S.E.	Mean for both seasons: No. of samples	Mean for both seasons: Mean	Mean for both seasons: S.E.
			Inorganic phosphorus: mg. per 100 ml. serum						
1	4 to 5 yr.	8	4.34	± .23	4.12	± .27	16	4.23	± .17
2	9 to 11 yr.	7	3.95	± .13	3.64	± .11	14	3.79	± .08
3	16 to 20 yr.	4	3.50	± .22	3.64	± .29	8	3.57	± .17
	Mean for all ages	19	4.02	± .13	3.84	± .14	38	3.93	± .10
	Range	....	.....		.....		....	3.00 to 5.78	
			Alkaline phosphatase activity: Bodansky units per 100 ml. serum						
1	4 to 5 yr.	8	8.1	± .6	5.8	± 4.6	16	6.9	± .5
2	9 to 11 yr.	7	5.8	± .2	5.0	± .3	14	5.4	± .2
3	16 to 20 yr.	4	5.9	± .5	3.9	± .6	8	4.9	± .5
	Mean for all ages	19	6.8	± .5	5.1	± .3	38	5.9	± .3
	Range	....	.....		.....		....	3.2 to 10.9	
			Calcium: mg. per 100 ml. serum						
1	4 to 5 yr.	8	12.18	± .17	12.41	± .22	16	12.33	± .10
2	9 to 11 yr.	7	12.03	± .15	12.59	± .13	14	12.31	± .13
3	16 to 20 yr.	4	13.42	± .28	12.59	± .21	8	13.01	± .23
	Mean for all ages	19	12.39	± .05	12.54	± .06	38	12.48	± .09
	Range	.....	.....		.....		.....	11.49 to 14.20	

*Standard error.

on the levels of calcium and magnesium, the mean values and the ranges for these factors obtained from all samples from non-

sium in the saddle animals than in the draft animals. A greater individual variation was observed in inorganic phosphorus in

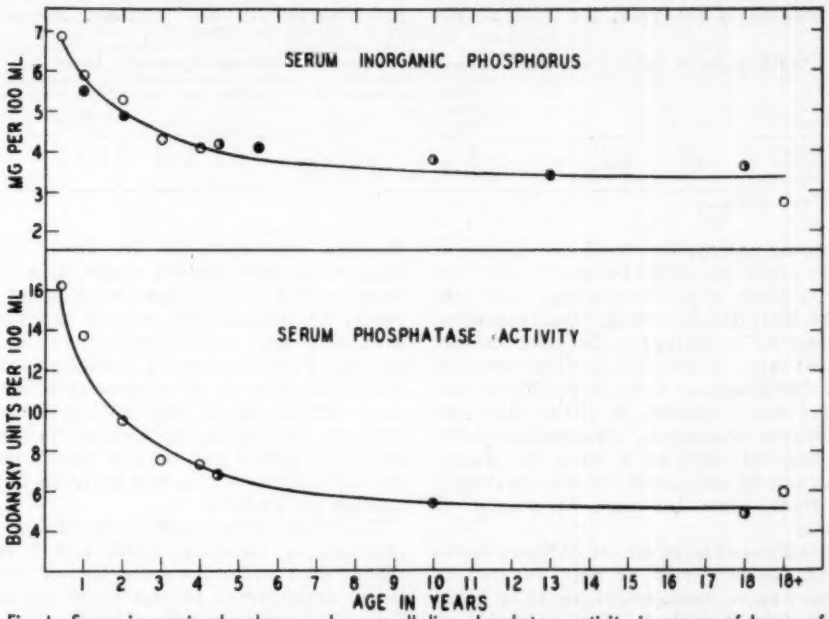

Fig. 1—Serum inorganic phosphorus and serum alkaline phosphatase activity in groups of horses of different ages in different geographic locations. Open circles = Vermont horses : mean of values for four seasons; Black circles = Montana horses : mean of values for one season; Half black circles = Maryland horses : mean of values for two seasons.

pregnant, nonlactating animals at each locality are compared in table 5.

DISCUSSION AND SUMMARY

Data on the levels of inorganic phosphorus, alkaline phosphatase activity, calcium, and magnesium in serum of apparently healthy horses have been assembled to indicate the relative importance, under conditions prevailing for the animals studied, of breed, age, season, general method of feeding, and lactation in effecting variations in the factors studied.

Breed.—A comparison of saddle-type and draft-tye animals from 1 to 8 years of age was permitted by the data on horses in Montana. Levels of inorganic phosphorus, calcium, and magnesium did not differ in the two types of animals between the ages of 2 and 8 years but, in yearlings, there was a tendency toward slightly higher mean levels of inorganic phosphorus and magne-

the saddle animals at all ages, although the mean levels in the two types at any age above 1 year differed little.

Age.—Age is obviously the greatest factor in the normal variations in both inorganic phosphorus and phosphatase activity. The mean values for the various age groups of horses at the three geographic locations, irrespective of methods of feeding or of season (fig. 1), indicate that the mean inorganic phosphorus declines from about 6.9 mg. per 100 ml. of serum at 5 months of age to approximately 4.2 mg. at 4 years, after which a further decline with advancing age proceeds very slowly. The mean alkaline phosphatase activity declines from about 16 units per 100 ml. at 5 months to about 6.5 units at 6 years, after which any further decline due to age seems negligible.

The data show the calcium level not to be influenced by age. Magnesium, however, in Vermont horses tends to be slightly lower

in the animals under 2 years of age than in those 2 years old or older.

Season.—Although analyses were made on the same animals in Vermont at different seasons of the year, the data do not permit an interpretation of the effects of season alone, since the feeding system was altered along with the season. The data on the Maryland horses, however, represent two seasons' samplings on the same animals without any change in feeding practice. Here, there appears to be no significant difference due to season in either inorganic phosphorus or calcium. Phosphatase activity, however, showed a drop in March, which can be accounted for only as resulting from some influence of season or climate.

Nutrition.—The effects of different methods of feeding, such as may be used in ordinary horse management, are to be found in the data from both Montana and Vermont horses.

In the Montana horses, range-fed animals appeared to differ from those of the same age which were fed grain and hay—principally in having a lower mean level of serum calcium, *i.e.*, 11.50 mg. per 100 ml. of serum as compared with 12.09 mg.

Sensitivity of the inorganic phosphorus level to changes in feeding is well demonstrated by the data obtained in four successive seasons on the Vermont horses. Mean levels found in October for each age group, except that of the aged animals, suggest a low phosphorus content of the fall pasture. This initial low serum level rose with the winter feeding of grain and hay to a level in January which was apparently unchanged in the spring after the animals were put out to pasture. The increase over the spring level which was observed in August can doubtless be attributed to the effects of the phosphate treatment given to the pastures in June.

Seasonal changes in serum phosphatase activity shown by each age group of the Vermont horses are considered to be largely effects of changes in diet, although the data on Maryland horses indicate that season also has some influence on phosphatase activity. The decreased phosphatase activity observed in the Vermont horses in January was succeeded in April, which represented early pasture season, by a higher mean level in every age group except the aged. In August, the general trend was toward a still higher level than in the spring. Further, it seems significant that 5 individual animals of various ages, which were not allowed to graze during the summer for one reason or another, failed to show this spring and summer rise in phosphatase activity which was observed in the animals on pasture.

TABLE 5—Serum Calcium and Serum Magnesium in Horses in Different Geographic Locations

Location	No. of sample	Age	Calcium (mg./100 ml. serum) Mean	S.E.*	Range	Magnesium (mg./100 ml. serum) Mean	S.E.	Range
Montana	98	1 to 16 yr.	11.89	± .06	10.73–13.11	2.03	± .02	1.61–2.37
Vermont	107	5 mo. to 22 yr.	12.07	± .07	9.97–13.42	1.93	± .05	1.25–2.65
Maryland	38	4 to 20 yr.	12.48	± .09	11.49–14.20	.....		.....

*Standard error.

Lactation.—Observations made on 17 pregnant or lactating mares and 8 open mares, kept under the same feeding conditions, demonstrate in the brood mares a drop in serum inorganic phosphorus about the time of parturition and a marked drop in calcium together with an increase in phosphatase activity with advanced lactation.

These data are belived to demonstrate the general trend and extent of variations in inorganic phosphorus, alkaline phosphatase activity, calcium, and magnesium which may occur in apparently healthy and sound animals with changing age, diet, season, and reproductive status.

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Comparison of the Potencies and Specificities of Human and Bovine Tuberculins

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McINTOSH AND KONST,[1] working in Canada, have reported that intradermic tuberculin for cattle testing, prepared according to the method used in the laboratories of the U. S. Bureau of Animal Industry, is less specific when tested on guinea pigs than tuberculin prepared by methods used in the Division of Animal Pathology, Science Service, Department of Agriculture, Canada. The U. S. BAI tuberculin is derived from cultures of three strains of *Mycobacterium tuberculosis* var. *hominis*, while the Canadian tuberculin is derived from a strain of *Mycobacterium tuberculosis* var. *bovis*. The Canadian workers, with the use of bovine tuberculin, were able to distinguish more clearly between two groups of guinea pigs, one of which was sensitized to *M. tuberculosis* var. *bovis*, and the other to *Mycobacterium tuberculosis* var. *avium*, or to *Mycobacterium paratuberculosis*, than when they used the human tuberculin. However, the potency of the bovine type of tuberculin was not as high as that of the human type. Further, these workers found that purified protein derivative (P.P.D.) prepared from the two types had about the same specificity, and served about equally well in differentiating between the two groups of animals. Later work by these investigators[2] has shown that a number of strains of *M. tuberculosis* var. *bovis*, other than the one used in the earlier investigation yield more P.P.D. per liter of medium than that strain. The culture filtrates of all but one of these high-yielding strains after ten weeks of growth had final pH values of 6.8 to 7.6. The culture filtrates of strains characterized by low yields of P.P.D. had final pH values of 5.6 to 6.0.

For several years, workers in the BAI laboratories have been attempting to improve the specificity of the Bureau tuberculin by chemical, physical, and biological means. Included in these studies have been tests to determine whether or not specificities could be improved by the use of strains of *M. tuberculosis* var. *bovis*. This paper reports the results of these studies. The results in general confirm those of the Canadian workers, but they also indicate that the superiority in specificity of the bovine tuberculins studied seldom reaches statistical significance and is not consistent from one experiment to the next.

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EXPERIMENTAL

Human tuberculins used in these tests were produced from cultures grown on the BAI synthetic medium first described by Henley,[3] and the bovine tuberculins were produced from cultures grown on either the BAI medium or the medium used in Canada.[2] Purified protein derivative was prepared from the two types of tuberculins according to the method of Green.[4]

Tests with 0.05 ml. of the materials per injection were made on guinea pigs in order to study their specificity in a preliminary way. The pigs were artificially sensitized by means of a subcutaneous injection of 5 to 10 mg. (dry weight) per animal of heat-killed *M. tuberculosis* var. *bovis*, or *M. tuberculosis* var. *avium*, suspended in mineral oil. Subsequent tests with 0.2 ml. injections were carried out on cattle artificially sensitized by means of the intraperitoneal injection of 3 Gm. per animal of similarly treated bacilli of various acid-fast types. (For further details see below.)

Purified protein derivative at a concentration of 1.5 mg. of dry weight per milliliter of solution was considered to be equivalent in potency on homologously sensitized animals to the issue strength for regular field testing of BAI intradermic tuberculin (¼ O.T.). The reason for this was that this concentration of P.P.D. was used in 1946 as the issue strength of the English P.P.D. tuberculin, according to Green.[4]

The bovine tuberculins were used at higher concentrations than the human tuberculins on homologously sensitized animals because they were known to be less potent than the latter.

The multiple testing method of Johnson[5] was used in this work. Experimental designs for testing and the details of the methods used for

the analysis of the data were those described by Wadley.[6,7]

Further details concerning the materials tested and the test animals are given under the description of the individual experiments.

Experiment 1A—June, 1948.—Materials.—The materials used were: (1) standard, a pool of 5 ml. aliquots from five serials of BAI intradermic tuberculin, all less than 1 year of age; (2) BAI P.P.D. prepared from the standard by the method of Green[4] modified to accomplish the precipitation of the active material from the finished BAI tuberculin instead of from the culture filtrate and to purify the precipitate further by reprecipitation; (3) Canadian serial Q tuberculin—obtained through the courtesy of the Animal Disease Research Institute, Hull, Quebec, and derived from the bovine strain 110 (furnished at ½ O.T. strength); and (4) serial Q P.P.D., prepared by Green's method from material (3). The materials were tested at three concentrations each (5-fold dilutions) on each animal. Placement of injections was random in nature.

Test Animals.—Six guinea pigs previously sensitized with 5 to 10 mg. (dry weight) of heat-killed *M. tuberculosis* var. *bovis* per animal were used as homologously sensitized animals, and 6 similarly sensitized to *M. tuberculosis* var. *avium* were used as heterologously sensitized animals.

The results of the experiment are shown in table 1A. Average diameters of reactions on each group of guinea pigs are given. The relative potencies of the different materials are used in the calculation of specificity factors.

Experiment 1B—June and July, 1948.—Materials.—The materials were identical with those of experiment 1A. Four concentrations of each material (4-fold dilutions) were tested on each animal.

Test Animals.—Nine head of cattle were each sensitized to *M. tuberculosis* var. *bovis* one month before being used as homologously sensitized animals. Nine head of cattle similarly sensitized to *M. paratuberculosis* served as heterologously sensitized animals.

The results are shown in table 1B. Averages of the increases in skin thickness resulting from the injections are given.

Experiment 2A—October, 1948.—Materials—The materials were: (1) standard, a pool of aliquots from ten BAI serials all less than 1 year old; (2) BAI P.P.D. prepared from the standard pool; (3) bovine tuberculin—a pool of aliquots from two batches of bovine tuberculin prepared in the BAI laboratory and derived from bovine culture 110 (differed from the Canadian serial used in experiments 1A and 1B only in that the storage period of one year in the cold room was omitted); and (4) bovine P.P.D. prepared from material (3).

Three concentrations at fivefold dilutions were tested on each animal.

Test animals.—Four guinea pigs were sensitized to *M. tuberculosis* var. *bovis* in the same manner as in experiment 1A and 4 sensitized to *M. tuberculosis* var. *avium* were used.

The results are shown in table 2A.

Experiment 2B—March, 1949.—Materials.—The materials were: (1) standard, a pool of aliquots from 14 BAI serials all less than 1 year old; (2) a pool of 13 BAI serials 1 to 2 years old; (3) a pool of 11 BAI serials 2 to 3 years old; (4) a pool of 13 BAI serials 5 to 6 years old; (5) a pool of 100 ml. aliquots from culture

TABLE 1A—Comparison, on Guinea Pigs, of the Specificity of a Pool of Aliquots from Five Serials of BAI Intradermic Tuberculins as a Standard, with the Specificities of a BAI P.P.D., Canadian Bovine Tuberculin, and a P.P.D. Made from Canadian Tuberculin (Six Animals in Each Sensitization)

Material	Dosage	Average diameter of reactions (mm.) on sensitized animals		Relative potency on sensitized animals		Specificity factor(a)
		Bovis	*Avium*	*Bovis*	*Avium*	
	O.T.					
	1/100	14.0	11.5			
Standard	1/500	11.0	9.5	1.0	1.0	1.0
	1/2,500	8.5	7.2			
	mg./ml.					
BAI	0.06	13.9	7.8			
P.P.D.	0.012	10.8	5.8	0.8	0.06	13.0**
	0.0024	7.7	4.7			
	O.T.					
Bovine	1/50	14.2	9.5			
Serial Q	1/250	11.5	5.9	0.6	0.14	4.0*
	1/1,250	8.6	4.1			
	mg./ml.					
Bovine	0.06	14.7	8.3			
Serial Q	0.012	11.6	4.2	1.7	0.06	28.0**
P.P.D.	0.0024	9.7	4.0			

*Statistically significant. **Highly significant. (a) 3.8 is required for statistical significance and 6.0 for high significance.

filtrates used in the manufacture of 11 BAI subserials, all before evaporation but after being phenolized and filtered through a Chamberland filter, less than 1 year old; (6) a pool of aliquots from the sublots of material (5) after evaporation, phenolization, and filtration, less than 1 year old; (7) an aliquot from BAI serial 904 before filtration, less than 1 year old; (8) an aliquot from same after filtration, less than 1 year old; (9) BAI P.P.D. prepared from the standard; (10) pool of aliquots from bovine tuberculins produced in BAI laboratory and from Canadian serials Q and 18; and (11) bovine P.P.D. prepared from the pool of material (10).

The materials were each tested at four concentrations (4-fold dilutions) on each animal.

Test Animals.—Ten cattle sensitized to *M. tuberculosis* var. *bovis* in the same manner as in experiment 1B and 10 sensitized to *M. paratuberculosis* were used.

The results are shown in table 2B.

TABLE 1B—Comparison, on Cattle, of the Specificities of the Same Materials Included in Table 1A (9 Cattle in Each Sensitization)

Material	Average increase in skin thickness (in mm.) at indicated dosages				Relative potency		Specificity factor(a)
	On *bovis*-sensit. cattle		On *paratub.*-sensit. cattle		On *bovis*	On *paratub.*	
	O.T.		O.T.				
	1/40	10.5	1/20	5.2			
Standard	1/160	6.3	1/80	3.7	1	1	1
	1/640	4.5	1/320	1.6			
	1/2,560	2.6	1/1,280	1.2			
	mg./ml.		mg./ml.				
	0.15	9.0	0.30	3.4			
BAI	0.0375	6.4	0.075	2.8	0.72	0.45	1.6
P.P.D.	0.009375	4.1	0.01875	1.8			
	0.0023475	2.2	0.004675	1.1			
	O.T.		O.T.				
	1/20	6.6	1/10	3.2			
Bovine	1/80	4.1	1/40	1.4	0.13	0.08	1.4
Serial Q	1/320	3.4	1/160	1.1			
	1/1,280	1.8	1/640	0.2			
	mg./ml.		mg./ml.				
Bovine	0.15	9.8	0.30	4.4			
Serial Q	0.0375	6.0	0.075	1.9	0.87	0.34	2.6
P.P.D.	0.009375	4.8	0.01875	0.9			
	0.0023475	2.5	0.004675	0.5			

(a) In these experiments 3.76 is required for statistical significance.

TABLE 2A—Comparison, on Guinea Pigs, of the Specificity of a Pool of Aliquots from Ten Serials of BAI Intradermic Tuberculins as a Standard, with the Specificities of a BAI P.P.D., a Pool or Aliquots of Different Batches of Bovine Tuberculin, and a Bovine P.P.D. (4 Animals in Each Sensitization)

Material	Average diameter of reactions (in mm.) at indicated dosages				Relative Potency		Specificity factor(a)
	On *bovis*-sensit. animals		On *avium*-sensit. animals		On *bovis*	On *avium*	
	O.T.		O.T.				
	1/100	14.1	1/50	12.4			
Standard	1/500	11.0	1/250	8.9	1.0	1.0	1.0
	1/2,500	8.8	1/1,250	6.8			
	mg./ml.		mg./ml.				
BAI	0.06	10.8	0.12	8.9			
P.P.D.	0.012	9.4	0.024	7.0	0.3	0.2	1.5
	0.0024	8.5	0.0048	6.2			
	O.T.		O.T.				
Bovine	1/50	13.4	1/25	10.8			
Tuberculin	1/250	11.2	1/125	7.7	0.4	0.2	2.0
	1/1,250	8.6	1/625	7.6			
	mg./ml.		mg./ml.				
Bovine	0.06	14.8	0.12	11.6			
P.P.D.	0.012	11.2	0.024	9.2	1.0	0.7	1.4
	0.0024	10.0	0.0048	6.7			

(a) In these experiments, 3.1 is required for significance.

Experiment 3—May, 1949.—Materials.—The materials used were: (1) standard, same as that used in experiment 2A; (2) P.P.D. prepared from pool of aliquots from the two bovine tuberculins produced in the BAI laboratory, as mentioned in experiments 2A and 2B and Canadian serial 18.

The materials were tested at four concentrations (3-fold dilutions) on four skin areas of each animal, making four replications per animal.

Test Animals.—They were the same as in experiment 2B.

The results are shown in table 3.

Experiment 4A—November, 1948.—Materials.—The materials used were: (1) standard, same as used in experiment 2A; (2) aliquot portions of culture filtrate from 4 cultures of *M. tuberculosis* var. *bovis* strain "calf" obtained through the courtesy of Dr. H. E. Ottosen of the Statens Veterinaire Serumlaboratorium, Kobenhavn, Denmark; (3) similar filtrate from *bovis* strain "Otto" from Denmark; and (4) culture filtrate from single culture of *bovis* strain "udder" from Denmark.

Two concentrations at tenfold dilutions of each material were tested on each animal.

Test Animals.—Four guinea pigs sensitized to *bovis* and 4 to *avium* varieties of *M. tuberculosis* were used.

The results are shown in table 4A.

Experiment 4B—August, 1949.—Materials.—The materials used were: (1) standard, a pool of aliquots from 34 BAI serials produced in 1947, 1948, and 1949; (2) a pool of aliquots from filtrates of 24 cultures of *M. tuberculosis* var. *hominis*, strain PN (cultures selected at random growing on three different batches of mediums and each originating from different seed cultures); (3) aliquots from filtrates of 24 cultures of *hominis* strain C; (4) aliquots from filtrates of 24 cultures of *hominis* strain DT; (5) aliquots from 20 cultures of Canadian *bovis* strain 110; (6) aliquots from 25 cultures of BAI *bovis* strain 90; (7) aliquots from 21 cultures of BAI *bovis* strain 10; (8) aliquots from 22 cultures of Denmark *bovis* strain "udder"; (9) aliquots from 18 cultures of Denmark *bovis* strain "calf"; and (10) aliquots from 18 cultures of Denmark *bovis* strain "Otto."

Each material was tested at four concentrations (1.5-fold dilutions) on each animal.

Test Animals.—To each of the following five

TABLE 2B—Comparison, on Cattle, of the Specificities of a Number of Pools of Aliquots of Serials of BAI Intradermic Tuberculins with the Specificities of a BAI P.P.D., a Pool of Aliquots of Bovine Tuberculins, and a Bovine P.P.D. (10 Cattle in Each Sensitization)

Material	Average increase in skin thickness (in mm.) at indicated dosages								Relative potency		Specificity factor(a)
	On *bovis*-sensit. cattle				On *paratub.*-sensit. cattle				On *bovis*	On *paratub.*	
	O.T.				O.T.						
	1/16	1/64	1/256	1/1,024	1/4	1/16	1/64	1/256			
BAI—1 yr.	11.4	6.4	4.0	1.6	10.6	6.5	3.6	1.7	1.0(b)	1.0	1.0
BAI—1-2 yr.	9.8	6.2	4.0	1.6	7.9	4.2	3.1	1.3	0.81	0.46	1.76
BAI—2-3 yr.	7.4	5.0	2.8	1.9	6.2	3.7	2.1	1.5	0.43	0.26	1.65
BAI—5-6 yr.	9.5	5.6	3.2	2.5	7.3	4.4	2.6	1.2	0.71	0.39	1.82
BAI filtrate before evap. (c)	8.7	5.0	2.8	2.1	6.2	3.8	1.6	0.8	1.36	0.47	2.91**
BAI filtrate after evap.	11.8	8.2	4.1	2.5	7.6	4.7	2.3	1.0	1.41	0.41	3.48**
BAI serial No. 904A (see text)	11.2	7.7	3.9	2.3	7.3	3.9	2.3	1.2	1.20	0.34	3.56**
904B	11.1	7.8	4.0	2.9	7.5	4.5	2.6	1.6	1.32	0.42	2.14*
	O.T.				O.T.						
	1/8	1/32	1/128	1/512	1/2	1/8	1/32	1/128			
Bovine tuberculin	7.4	4.5	3.0	1.6	5.5	3.1	1.8	1.0	0.21	0.093	2.26*
	mg./ml.				mg./ml.						
	.38	.094	.024	.006	1.5	.38	.094	.024			
BAI P.P.D.	7.4	5.0	3.4	2.2	5.5	2.7	1.7	1.1	0.56	0.17	3.29**
Bovine P.P.D.	7.4	4.5	2.6	2.0	4.3	2.4	1.9	1.1	0.42	0.11	3.82**

(a) In these experiments, 2.12 is required for statistical significance and 2.71 for high significance.

(b) The first BAI pool is arbitrarily considered to have a potency of 1 and a specificity of 1 in both sensitizations. Any one of the other BAI pools or tuberculins could have been assumed with justification to have potencies and specificities of 1.

(c) The concentrations of this material were in all cases 2½ times as dilute, as column headings indicate.

*Statistically significant. **Highly significant.

types of acid-fast bacilli, 10 cattle were sensitized: (1) *M. tuberculosis* var. *bovis*, homologous for bovine and human material and heterologous for others; (2) *M. tuberculosis* var. *hominis*, homologous for human materials and heterologous for others; (3) *M. tuberculosis* var. *avium*, heterologous for all materials; (4) *M. paratuberculosis*, heterologous for all; and (5) *Mycobacterium phlei*, heterologous for all.

The results are shown in table 4B in the form of the relative potencies and specificity factors of the different materials tested. Limited space prevented the inclusicn of average reactions.

scale production of tuberculin by the Bureau is to make available for use in eradication work a diagnostic agent for the detection of animals sensitized to *M. tuberculosis* var. *bovis*, the first requisite of the agent is satisfactory potency. Low potency tuberculins are less likely to give reactions on poorly sensitized animals than are high potency tuberculins. Results of field work reported by McIntosh and Konst[1] and unpublished results of field work carried out in 1939 in the Dakotas by BAI veterinarians show conclusively that more tuberculous

TABLE 3—Comparison, on Cattle, of the Specificity of a Pool of Aliquots of Ten Serials of BAI Intradermic Tuberculins as a Standard with the Specificity of a Bovine P.P.D. Each Concentration of Each Material Was Tested Four Times on Each Animal (10 Animals in Each Sensitization)

Material	Average increase in skin thickness (in mm.) at indicated dosages				Relative potency		Specificity factor(a)
	On *bovis*-sensit. cattle		On *paratub*-sensit. cattle		On *bovis*	On *paratub*	
	O.T.		O.T.				
	1/12	10.5	1/4	7.7			
Standard	1/36	7.1	1/12	5.2			
	1/108	4.4	1/36	3.2	1	1	1
	1/324	2.5	1/108	2.0			
	mg./ml.		mg./ml.				
	2.0	7.2	6.0	3.7			
Bovine	0.67	4.6	2.0	2.3			
P.P.D.	0.22	3.1	0.67	1.5	0.30	0.14	2.2
	0.07	2.0	0.22	0.8			

(a) In these experiments, 3.5 is required for statistical significance.

DISCUSSION

From the data presented, it seems probable that tuberculins derived from cultures of *M. tuberculosis* var. *bovis* are more specific than the regular BAI intradermic tuberculins. However, the potencies of the bovine tuberculins in sensitized animals were only ⅛ to ¾ those of the BAI tuberculins. Since the chief purpose of the large-cattle are detected by the regular BAI tuberculins than by the less potent bovine tuberculins.

The lack of consistency in the results from the specificity comparisons in the present study is shown by the following: Of the 31 comparisons between bovine and BAI tuberculins, the former was significantly superior in 2 instances, superior but

TABLE 4A—Comparison, on Guinea Pigs, of the Specificity of a Pool of Ten Serials of BAI Intradermic Tuberculins as Standard with the Specificities of Tuberculins Derived from Three Different Strains of Bovine Tubercle Bacilli (4 Animals in Each Sensitization)

Material	Dosage	Average diameter of reactions (mm.) in sensitized animals		Relative potency in sensitized animals		Specificity factor(a)
		Bovis	*Avium*	*Bovis*	*Avium*	
	O.T.					
Standard	1/100	13.1	7.5	1.0	1.0	1.0
	1/1,000	9.3	6.6			
"Calf"	1/50	11.4	6.9	0.2	0.1	2.0
	1/500	7.9	5.6			
"Otto"	1/50	13.0	7.4	0.5	0.3	1.7
	1/500	9.5	6.2			
"Udder"	1/50	11.9	6.9	0.2	0.1	2.0
	1/500	7.7	5.2			

(a) In this experiment, 4.2 is required for statistical significance.

not significantly so in 13, equal to the BAI tuberculins in seven cases, and inferior in nine.

Some of the above figures would have been changed by changing the BAI tuberculin selected as a standard. If any one of the last four BAI tuberculins listed in table 2 had been chosen as the standard, there would have been no superiority in specificity of the bovine tuberculins over the standard in that experiment, and the number of instances in which they showed up as significantly superior for all experiments would have been only one instead of two. This fact suggests that results of experiments involving only a limited number of batches of each type of tuberculin should be interpreted with caution.

A few tests were carried out on the two types of P.P.D. (BAI and bovine). The P.P.D. preparations were more specific than the BAI tuberculins, but those from the two types of tuberculins were not significantly different. Their superiority over the BAI tuberculins was not consistent from test to test. This confirms, in general, other experiences. Although P.P.D. tuberculins are used extensively in England, Green[4] makes no claim that they are better for practical use than tuberculins prepared in the ordinary ways. Baisden, Johnson, and Larsen[8] have reported the results of many tests, on artificially sensitized cattle, which compared the specificities of BAI human tuberculins and precipitates derived from them. Those precipitates were essentially the same as the BAI P.P.D. used in this work. The precipitates were not more specific than the parent materials.

The dosages of the two types of P.P.D. used in these experiments were based on the fact that formerly in England[4] the issue strength for field testing was 1.5 mg. of dry weight per milliliter of solution as pointed out previously. It was, therefore, assumed for the purpose of comparison that 1.5 mg. dry weight of P.P.D. per milliliter was equivalent to the issue strength of BAI intradermic tuberculins of ¼ O.T. It can be seen from the tables that in only two out of nine cases, in the present experiments, did a P.P.D. equal or exceed the standard in potency in animals sensitized to *M. tuberculosis* var. *bovis*. Thus, the dose of active material per unit volume of P.P.D. solution is probably less than that of BAI tuberculin, and in field tests the P.P.D. would be less likely than the BAI tuberculins to react in poorly sensitized animals.

Unexpected results were obtained in experiment 4B (table 4B) in that the bovine tuberculins in general appeared to distinguish better between *M. tuberculosis*, var. *bovis* sensitization and *M. tuberculosis*, var. *hominis* sensitization than between *M. tuberculosis*, var. *bovis* on the one hand and *M. paratuberculosis*, *M. tuberculosis*, var. *avium*, and *M. phlei* sensitizations on the other. The experience of many workers,

TABLE 4B—Comparison, on Cattle, of the Specificity of a Pool of Aliquotes of Thirty-Four Serials of BAI Intradermic Tuberculins with the Specificities of Culture Filtrates from Cultures of Three BAI Human Strains, One Canadian Bovine, Two BAI Bovine, and Three Danish Bovine Strains of Tubercle Bacilli (10 Cattle in Each Sensitization)

	Relative potencies on various sensitizations					Specificity factors						
Material	*Bovis*	*Hom.*	*Paratub.*	*Avium*	*Phlei*	B/J	B/A	B/P	B/H	H/J	H/A	H/P
Standard	1.00	1.00	1.00	1.00	1.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Human PN	1.02	0.92	1.02	1.17	1.65	1.0	0.9	0.6	1.1	0.9	0.8	0.6
Human C	1.22	1.22	1.12	1.69	1.68	1.1	0.7	0.7	1.0	1.1	0.7	0.7
Human DT	1.46	1.40	1.21	1.56	1.85	1.2	0.9	0.8	1.0	1.2	0.9	0.8
Canadian bovine No. 110	0.58	0.34	0.45	0.52	0.34	1.3	1.1	1.7	1.7	0.8	0.7	1.0
BAI bovine No. 90	0.42	0.20	0.24	0.33	0.31	1.8	1.3	1.4	2.2*	0.8	0.6	0.6
BAI bovine No. 10	0.46	0.14	0.29	0.32	0.32	1.6	1.4	1.4	3.3†	0.5	0.4	0.4
Danish bovine "Udder"	0.17	0.08	0.22	0.27	0.18	0.8	0.6	0.9	2.2*	0.4	0.3	0.4
Danish bovine "Calf"	0.44	0.39	0.29	0.43	0.43	1.5	1.0	1.0	1.1	1.3	0.9	0.9
Danish bovine "Otto"	0.74	0.37	0.51	0.98	0.56	1.5	0.8	1.3	2.0*	0.7	0.4	0.7
Factor required for statistical significance						2.1	1.9	2.3	2.0	1.8	1.7	1.8
Factor required for high significance						2.7	2.4	3.0	2.5	2.1	2.1	2.3

*Statistically significant. †Highly significant.

e.g., that of Johnson, Larsen, Henley, and Groth[9] and Green[4] has indicated that opposite results would ordinarily be expected. Infections caused by *M. tuberculosis* var. *bovis* and *M. tuberculosis* var. *hominis* are generally believed not to be distinguishable by the intradermic tuberculin test.

SUMMARY

Bureau of Animal Industry intradermic tuberculins and bovine tuberculins have been compared for specificity, or ability to distinguish animals sensitized to *Mycobacterium tuberculosis* var. *bovis* from animals sensitized to the following acid-fast microörganisms: *Mycobacterium tuberculosis* var. *hominis*, *Mycobacterium tuberculosis* var. *avium*, *Mycobacterium paratuberculosis*, and *Mycobacterium phlei*. Purified protein derivative (P.P.D.) preparations from bovine and human tuberculins were also compared with each other and their parent materials. Test animals were guinea pigs and cattle artificially sensitized to the heat-killed bacilli.

In general, the bovine tuberculins used appeared to be more specific than the human BAI tuberculins, but the superiority rarely reached statistically significant levels and was not consistent from one experiment to another. Also, bovine tuberculins were found to be only about ⅛ to ¾ as potent as BAI tuberculins when tested on animals sensitized to *M. tuberculosis bovis*. Hence, they were less desirable for routine use because of the importance of having a potent diagnostic agent for detecting poorly sensitized animals in the field.

In one experiment in which several BAI tuberculins were tested, significance of superiority in specificity of the bovine tuberculins and the P.P.D. preparations, as compared with the BAI tuberculins, depended on which of the particular BAI tuberculins was selected as the standard. These results suggest that it is necessary to observe caution in drawing conclusions from tests of only a few batches of each type of tuberculin.

Unexpected results were obtained in an experiment in which tuberculins derived from a number of *bovis* strains were compared with BAI tuberculin on *bovis*, *hominis*, *avium*, *paratuberculosis*, and *phlei* sensitization. All instances of statistically superior specificity shown by the bovine tuberculins in the experiment were the result of their distinguishing between *bovis* and *hominis* sensitizations.

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Effects of Low Temperature on the Eggs of the Common Liver Fluke (Fasciola Hepatica) in Beef Livers

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UNDER FEDERAL meat inspection, beef livers infected with flukes are regarded as unfit for human food and are, in fact, condemned for this purpose when they contain even a single fluke or show lesions characteristic of this parasitic infection. Because of the possible danger of spreading liver flukes to domestic ruminants, infected livers are not even released for fish food unless they have been denatured and refrigerated so as to destroy life of the fluke eggs.

In the past, fluke-infected livers were required to be held at a temperature of not higher than 10 F. for not less than forty-eight hours.[7] This requirement was based on laboratory tests made by the Zoological Division many years ago. Those tests showed that this holding period at the specified temperature would destroy life in the fluke eggs. A few years ago, the question of the destruction of liver fluke eggs in beef livers was reopened, largely for the purpose of determining whether temperatures higher than 10 F. for longer than forty-eight hours could be substituted for the required refrigeration. In the course of these investigations, the required holding period of 10 F. also was reinvestigated to determine the effects of this temperature on fluke eggs in livers packed in boxes and insulated, therefore, against the low temperatures for considerable periods. The results of these investigations, which form the basis of a modified requirement under federal meat inspection, are briefly presented in this paper.

WORK OF PREVIOUS INVESTIGATORS

It has long been known that low temperatures retard development of the eggs of *Fasciola hepatica*. Thomas[6] found that, when eggs were kept at temperatures of 71.6 to 78.8 F., miracidia developed in fourteen days; whereas, it took two to three months at 60.8 to 64.4 F., and no development took place below 50 F. That eggs could survive as long as five months at 42.8 to 51.8 F. and then complete development at 59 F. was reported by Clunies Ross and McKay.[1] Krull[2] reported eggs remaining undeveloped but viable after more than two and one-half years under refrigeration at 35.6 to 50 F.

According to the investigations of Mattes,[4] eggs were capable of surviving short periods of exposure at 26.6 to 28.4 F. but were killed when exposed twenty-four to forty-eight hours at temperatures below 19.4 F. Shaw and Simms,[8] however, found newly collected eggs viable after exposure at 10 F. for twenty-four hours.

The foregoing observations were made on eggs removed from the liver or recovered from the feces and therefore not insulated against exposure by liver tissue and packaging, as in the present study. Luhrs,[3] in fact, suggested that temperatures below 19.4 F. or long exposure at 19.4 F. were required to kill fluke eggs insulated in piles of manure.

MATERIALS AND METHODS

Preliminary trials to determine a killing temperature for the eggs of *F. hepatica* were made by placing the eggs in 2-dram vials of water in the freezing unit of a refrigerator. The desired temperature was regulated as closely as possible and recorded by means of a maximum-minimum thermometer. Eggs were removed at 24-hour intervals and then incubated to determine whether they would develop.

Freezing experiments with infected beef livers obtained from a packingplant in Houston, Texas, were carried out in the packinghouse freezers. The procedure for handling the livers was the same as that normally followed at the packingplant. After removal of the gall bladder, the liver was slashed freely with a sharp knife before denaturing with finely powdered charcoal. Each liver was hung on a rack to chill for twenty-four hours in a cooler maintained at a temperature not higher than 40 F. The livers were then packed into wooden boxes and placed in the freezer. The containers used were of two sizes: (1) 30 in. long by 19 in. wide by 7 in. deep; and (2) 27 in. long by 18 in. wide by 17 in. deep. Ten to fifteen livers,

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or enough to weigh 100 lb., completely filled the shallow box; whereas, the same quantity of livers filled the deeper box slightly above the 7-in. level. Temperatures were recorded at two-hour intervals by means of a Fahrenheit thermometer.

For the tests, a heavily infected liver was chosen and placed in the center of each box so as to be surrounded by other livers. The boxes were stacked on wooden floats in the freezer, each tier containing three boxes arranged so that each had at least two sides uncovered. Three to eight tiers comprised a stack. During the experiments, boxes containing test livers were placed in various locations in the stacks so that some were on the bottom, some in the middle, and some on the top of the stack. At the end of the desired exposure period, the boxes were thawed in warm water, and the fluke eggs were collected from the test livers and washed free of debris. Several thousand eggs were then incubated in shallow water in Syracuse watch glasses at 89.6 F. Failure of the miracidia to develop and hatch after such incubation for at least sixty days was considered evidence that the eggs had been killed. Several thousand eggs were taken from each test liver before freezing and incubated in a like manner as a check on the original viability. The checks yielded miracidia in nine to twenty-three days in all cases.

RESULTS

In the preliminary tests, eggs of the common liver fluke stored in 39 different vials of water were killed when exposed for twenty-four hours to a minimum temperature of 19 F. or below. Eggs in 19 of 21 vials failed to survive twenty-four hours at 20 to 25 F. At 21 to 26 F. eggs in 14 of 25 vials were killed. At 22 F. and above, eggs in all 80 vials hatched, with the exception of those in one vial out of 36 stored at 23 to 28 F. Miracidia hatched from eggs incubated after six days' exposure to 26 F. and also after nine days' exposure to 30 F., when observations were stopped. These results indicated that, allowing a margin of safety, a minimum temperature somewhat below 19 F. should be maintained in order to destroy fluke eggs. For most of the experiments summarized in table 1, the freezer temperature was, therefore, maintained in this range as closely as possible. There were fluctuations because of difficulty experienced in controlling the temperature of the freezer.

In the tests, it was found that storing livers for two to five days at mean temperatures ranging from 15 to 17.5 F. failed to kill any appreciable number of eggs, as miracidia hatched out after nine to seventeen days of incubation. From one to a few hundred eggs from five out of 16 livers survived freezing for six to seven days at mean temperatures ranging from 13.2 to 16 F., the miracidia hatching out in twenty-

TABLE 1—Viability of Fasciola Hepatica Eggs in Beef Livers Exposed to Low Temperatures for Varying Periods

Infected livers exposed (No)	Days exposed (No.)	Freezer temperature (F.)			Livers with viable eggs (No.)	Miracidia hatched (No.)
		Maximum	Minimum	Mean		
2	2	10	7	8.7	2	thousands
2	3	12	8	10.2	2	thousands, 6
1	4	11	8	9.8	0	0
1	4	13	7	9.4	0	0
2	5	14	7	10.4	0	0
2	6	14	7	10.2	0	0
2	2	18	16	16.5	2	thousands
4	3	20	16	17.5	4	thousands
4	4	20	15	16.4	4	thousands
6	5	20	13	15.0	3	thousands
3	5	20	15	16.4	3	thousands
5	6	16	10	11.6	0	0
3	6	16	10	13.2	3	hundreds
2	6	20	13	15.3	0	0
3	7	16	11	13.7	2	hundreds, 1
1	7	18	12	15.2	0	0
2	7	18	15	16.0	0	0
2	8	18	12	14.8	0	0
2	8	19	14	16.4	0	0
3	8	20	14	16.9	0	0
4	8	20	15	17.2	2	1, 5
4	9	19	14	15.9	0	0
2	9	19	14	16.4	0	0
4	9	20	14	16.8	0	0
2	9	20	15	17.7	0	0

one to forty-nine days of incubation at 89.6 F. One and five eggs, respectively, in two of four livers survived eight days at a mean temperature of 17.2 F. and hatched after sixteen days' incubation, whereas all eggs were killed in eight days at temperatures of 14.8 to 16.9 F. All eggs were killed in four tests with 12 livers frozen for nine days at mean temperatures between 15.9 and 17.7 F. (table 1).

By using the procedures for freezing livers under the requirements formerly in effect, it was determined that thousands of fluke eggs in three out of four livers survived two and three days' exposure to mean temperatures of 8.7 and 10.2 F., respectively. Hatching occurred in nine to ten days. All eggs were killed, however, in livers frozen four days at mean temperatures of 9.4 and 9.8 F., in two livers frozen five days at 10.4 F., and in livers frozen six days at mean temperatures of 10.2 and 11.6 F., respectively (table 1).

The position of the box in the stack did not affect the results. It was observed, however, that livers were not completely frozen when exposed for less than three days at 10.2 F., when exposed for two to four days at mean temperatures of 16.4 to 17.5 F., or occasionally, when exposed for five days at 15.0 to 16.5 F. These observations show, therefore, that time must be allowed for all livers to reach a freezing temperature, in addition to the time required to kill the eggs after such temperature has been attained.

As a result of the tests recorded in this paper, the requirement for the freezing of livers containing flukes has been amended as follows:

> . . . Freezing shall be preceded by chilling the livers to a temperature not above 40° F. Livers packed in containers not more than 7 inches thick shall then be held for a period of not less than 10 days at a temperature not higher than 15° F. or for a period of not less than 5 days at a temperature not higher than 10° F. Livers packed in containers over 7 inches but less than 27 inches thick shall be held not less than 20 days at a temperature not higher than 15° F., or for not less than 10 days at a temperature not higher than 10° F. Freezing may be accomplished in the regular freezer in a properly separated compartment or receptacle held under division lock. . . .[7, 8]

SUMMARY

1) Eggs of the common liver fluke, *Fasciola hepatica*, were all killed by freezing in small vials of water for twenty-four hours at 19 F. or below. The eggs survived at least six days at 26 F. and nine days at 30 F.

2) Eggs survived in large numbers in two livers after two days' exposure at a mean temperature of 8.7 and in one out of two livers exposed for three days at 10.2 F.; whereas, all eggs were killed in livers frozen four days at mean temperatures of 9.4 and 9.8 F.; in livers frozen five days at 10.4 F., and in livers frozen six days at temperatures of 10.2 and 11.6 F.

3) Large numbers of eggs survived in all livers exposed two to four days at mean temperatures ranging from 16.4 to 17.5 F. The incidence of survival decreased until, on longer exposure, five or less eggs in two out of 11 livers survived eight days at a mean temperature of 17.2 F.

4) Eggs were killed in all livers frozen nine days at mean temperatures ranging between 15.9 and 17.7 F., when exposed under the experimental conditions described herein.

5) Based on these results, the requirements for freezing livers containing flukes have been amended to hold livers for not less than ten days at a temperature not higher than 15 F. or for a period of not less than five days at a temperature not higher than 10 F. The storage time at these temperatures is doubled for containers of liver over 7 in. but less than 27 in. thick.

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The Effect of Added Manganese in Feed on Various Mineral Components of Cattle Blood

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THE RELATIONSHIP of calcium and potassium to the disease of cattle commonly referred to as "wheat poisoning" has been investigated and reported in earlier publications.[1, 2] The possibility of a correlation between the disease and the manganese (Mn) content of wheat resulted from the work of Stuart, Harbaugh, and Dennis.[3] The concentration of Mn in the winter wheat grazed by beef cattle in west Texas was determined by these authors. Their average value of 77 parts per million (p.p.m.) of Mn is not considered to be excessively high, but they have speculated on the relationship of the Mn to the mineral metabolism of the animals.

The present investigation was initiated in an attempt to investigate the effect of Mn, in varying amounts, on the glucose, calcium, magnesium, iron, and potassium levels in the blood of cattle and to determine whether Mn is an etiological factor in wheat poisoning.

PROCEDURE

Five purebred Hereford cows, representative of the Texas Technological College beef cattle herd, were chosen for the experiment. The animals were maintained in a separate enclosure during the entire experiment, and each animal was confined to a separate stall while feeding, in order to control the amount of feed consumed. The diet consisted of 40 lb. of silage, 2 lb. of alfalfa, and 2 lb. of cottonseed meal per animal per day.

Research was begun in November, 1950, and normal blood values were established for each animal. A determination of the Mn content of each of the three components of the diet was made, and sufficient manganous sulfate added to raise the value to 75 p.p.m. of Mn. The silage was found to contain approximately 22.1 p.p.m. of Mn, the alfalfa averaged 31.5 p.p.m., and the cottonseed meal contained about 15.7 p.p.m. The first Mn level was set at 75 p.p.m. on the basis of the work done by Stuart and associates.[3] Six blood samples were taken from each animal at this level, the time between samples averaging seven days. Subsequently, the Mn content of the feed was raised to 100 p.p.m. and five samples were obtained from each animal. Sampling was continued at levels of 150 and 200 p.p.m. of Mn. The administration of Mn was discontinued in June, 1951, and the experiment was concluded in July of that year, after additional normal blood values were obtained.

Stock solutions of manganous sulfate were prepared such that 30 ml. of solution contained the requisite amount of the salt and elevated the Mn content of the feed to the desired levels. The solutions were transferred directly from 30-ml. containers to the silage each day.

Blood samples were obtained in all cases from the external jugular vein and divided into two portions. One portion, utilized in the determination of potassium, glucose, and iron, approximated 50 ml. in volume. Sodium oxalate was utilized as the anticoagulant. The second sample, necessary for the determination of calcium and magnesium, was collected and allowed to clot in two 15-ml. centrifuge tubes. Each analysis was begun within an hour of sampling. Glucose was determined immediately, and the separation of whole and clotted blood into plasma and serum, respectively, was effected at once in order to prevent alteration of potassium and calcium values. The analytical methods have been enumerated in previous papers.[1-4]

RESULTS AND DISCUSSION

The results of the analyses are shown in tables 1 and 2.

In none of the animals examined were there any visible indications of the onset of symptoms of tetany. The thinness and emaciation noted by Harbaugh and Dennis[1] were absent, as were any indications of abnormal excitability. Beyond a normal reluctance to submit to the insertion of the needle, the animals gave no evidence of hyperexcitability. On the contrary, the animals appeared to derive benefit from the experimental diet. Although no detailed data were collected regarding weight, physical

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The data in this paper have been taken from a thesis presented to the Division of Graduate Studies by Miss Fain in partial fulfillment of the requirements for the degree of master of science.

appearance, etc., examinations as the experiment proceeded indicated the over-all health of the animals to be good.

An examination of table 2 indicates that the concentration of magnesium in the blood serum of the animals exhibited a downward trend during the period in which the Mn level was held at 100 p.p.m. The decreased magnesium concentrations were apparent in the animals for about four weeks. The values increased sharply when the Mn level was raised to 150 p.p.m. and they remained at approximately normal concentrations thereafter. These low values

TABLE 1—Normal Blood Values for the 5 Experimental Animals*

Cow (No.)	Glucose (mg./100 cc. blood serum)	Ca (mg./100 cc. blood serum)	Mg (mg./100 cc. blood serum)	Fe (mg./100 cc. blood)	K (mg./100 cc. blood serum)
97	75.20	10.58	1.91	35.50	16.50
149	72.20	10.92	2.03	33.80	16.30
150	75.70	11.04	1.84	33.60	16.90
152	71.30	11.04	2.01	35.40	15.10
157	75.20	11.20	1.87	35.60	17.20

*The figures given are averages of seven determinations.

can not be considered to be indicative of tetany, since concentrations of the order of 0.145 mg. per 100 cc. of blood serum have been noted in tetany due to hypomagnesia.[5] However, the simultaneous decrease of magnesium in all of the animals at 100 p.p.m. of Mn, and the subsequent rise at 150 p.p.m., are considered noteworthy.

The lowered magnesium values are somewhat similar to results obtained by Blakemore and co-workers.[6] They determined that blood magnesium values fell during the first few weeks that cattle were turned out to pasture. The magnesium concentrations then began to rise and attained normalcy within a short time. They found that the injection of Mn solutions produced like results.

Ratios of calcium to magnesium have been calculated and are shown in table 2. It can be seen that the values at 75, 150, and 200 p.p.m. of Mn are in agreement with the value of 5.6 reported for normal cattle by Sjollema and Seekles.[7] The ratios at 100 p.p.m., although somewhat elevated, do not approach the value of 14.6 noted by the same authors in animals suffering from tetany.

It is possible that a disturbance of magnesium metabolism occurs at Mn concentrations of approximately 100 p.p.m. concentrations to which cattle might be subjected in grazing wheat. The question

TABLE 2—Comparison of the Average Blood Values of the 5 Experimental Animals at Manganese Levels of 75 to 200 Parts per Million*

Cow (No.)	Glucose (mg./100 cc. blood serum)	Ca (mg./100 cc. blood serum)	Mg (mg./100 cc. blood serum)	Fe (mg./100 cc. blood)	K (mg./100 cc. blood serum)	K : Ca	Ca : Mg
75 p.p.m. of Mn							
97	61.80	10.48	2.17	39.40	15.80	1.50	4.83
149	60.60	10.42	1.84	32.20	15.80	1.51	5.66
150	67.40	10.39	2.07	35.70	17.20	1.65	5.02
152	63.70	10.21	1.77	34.80	15.90	1.55	5.76
157	67.90	10.77	2.05	34.50	16.40	1.52	5.25
100 p.p.m. of Mn							
97	65.50	10.40	1.26	34.30	13.90	1.33	8.25
149	62.20	10.40	1.33	32.90	15.20	1.46	7.82
150	67.10	10.46	1.59	35.00	15.90	1.54	6.57
152	66.50	10.81	1.49	34.40	16.80	1.55	7.25
157	64.80	10.43	1.46	35.50	16.90	1.62	7.41
150 p.p.m. of Mn							
97	63.70	10.80	2.41	37.30	14.40	1.33	4.48
149	65.60	11.19	2.15	36.00	18.40	1.64	5.24
150	60.80	10.96	2.01	36.80	17.20	1.56	5.45
152	60.80	11.14	1.90	34.50	16.70	1.49	5.86
157	58.50	11.65	2.02	37.70	16.80	1.44	5.76
200 p.p.m. of Mn							
97	61.70	11.16	1.97	35.30	17.40	1.59	5.66
149	72.50	10.49	1.90	39.80	17.80	1.68	5.52
150	65.10	10.77	2.29	38.40	15.50	1.44	4.71
152	67.10	10.31	1.79	35.70	16.20	1.57	5.76
157	67.40	10.71	2.07	39.80	18.30	1.70	5.17

*The values given above are averages of eighteen determinations.

arises as to whether the low magnesium values would prevail if the Mn level were maintained at 100 p.p.m. over a long period. Lack of finances prevents further investigation of the problem at this time.

Since the Mn content of the feed was determined only once, there is the possibility that the concentration in the feed varied sufficiently from time to time to depress the magnesium values. However, it appears unlikely that such variations would occur only at one level of Mn intake. There is the further possibility that metabolic factors not considered in this investigation had some bearing on the depression of magnesium.

The overall-all depression of calcium metabolism, as reported by Reid and coworkers[8] in their studies of the mineral

metabolism of cattle, was not apparent in this study, although some slight variations in calcium values were obtained from time to time. The concentrations of glucose and iron appeared to remain within normal limits throughout the experiment. Certain increases in the plasma potassium are indicated in table 2. However, no consistent trend is apparent, and the variations can probably be attributed to a temporary release of potassium into the blood stream, such a condition being induced by the nervous reaction to the sampling procedure.

SUMMARY

1) No indications of wheat poisoning are shown by cattle which are fed manganese (Mn) in amounts varying from 75 to 200 parts per million (p.p.m.)

2) A decrease in blood magnesium becomes apparent at Mn levels of 100 p.p.m., but normalcy is attained at 150 p.p.m. The depression of magnesium is not clearly understood, and there is some question as to whether continued intake of Mn at a level of 100 p.p.m. would produce still lower values of magnesium, resulting eventually in tetany.

3) The addition of Mn to the diet, in the amounts given, appears to have no significant effect on the concentration or metabolism of glucose, iron, calcium, or potassium.

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Studies on Repeated Vaccination of Cattle with Brucella Abortus Strain 19. III. The Response of Vaccinated and Revaccinated Cattle to Conjunctival Exposure with a Virulent Strain of Brucella Abortus During the Third Gestation Period

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THE RESULTS OF field trials and controlled experiments recently reviewed[1, 2] permit the conclusion that calfhood vaccination with *Brucella abortus* strain 19 engenders a serviceable degree of resistance to exposure during the first gestation period. Despite the difficulty in establishing quantitative relationships from the data available from investigations conducted with relatively small numbers of animals, and under widely different conditions (e.g., different strains and different numbers of organisms used for challenge), there is some evidence that the resistance engendered by calfhood vaccination declines with the passage of time.[3, 4]

Studies recently reviewed by Meyer[5] indicate that soluble Brucella antigens, prepared by a variety of methods, produce resistance in experimental animals, particulary when they are inoculated with adjuvants which delay absorption by the tissues, thus prolonging the contact of antigen with the host cells. Another approach to the prolongation of immunogenic stimulation is offered by repeated vaccination (as with strain 19).

Traum[6] has discussed the conditions under which revaccination is practiced among California herds when infection appears among calfhood-vaccinated animals. He states that field trials are in progress with "favorable" results, although data are not presented.

Lawson[7] has presented the results in 14 infected field herds in Great Britain in which animals were given two doses of strain 19, and five in which three inoculations were made. Differences are not apparent when the abortion rates in these herds are compared with those of 28 herds in which only one vaccination was made.

A report[8] has been presented on a program of revaccination and improved sanitation in a large "problem" herd in which calfhood vaccination and regular testing had not eradicated brucellosis. Unfortunately, the data did not permit evaluation of the relative significance, in the eradication of infection, of revaccination and of the sanitary measures which were employed.

The present paper, the third in a series on revaccination of cattle, records the responses of groups of vaccinated and revaccinated cattle to exposure during the third gestation period, as well as those of unvaccinated cattle exposed in the same manner during the first, second, or third pregnancies.

MATERIALS AND METHODS

In November, 1945, 64 unvaccinated grade Holstein-Friesian heifer calves, of approximately the same age and negative to the agglutination test, were purchased from various sources. At approximately 8 months of age, the calves were vaccinated subcutaneously with 5 ml. of a suspension of *Br. abortus* strain 19, which had been freshly prepared in this laboratory according to the U. S. Bureau of Animal Industry (BAI) standards for this vaccine. The calves were then assigned at random to three groups of equal size. The heifers in group 1 were not treated again. Those in group 2 were revaccinated subcutaneously six months later, at the age of 14 months. The animals in group

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3 were revaccinated twelve months after the first vaccination, at the age of 20 months. The three groups mingled as a unit thereafter and made up herd 5.

The animals were bred for the first pregnancy by natural service when they were 15 to 18 months old, by artificial insemination or natural service for the second pregnancy, and by natural service for the third.

Fifteen unvaccinated yearling heifers were added to the herd, to serve as controls, at the termination of the first pregnancies of the vaccinated animals. Four of these control heifers were bred so that they were in the second trimester of their first pregnancy at the time most of the remainder of the herd was calving for the second time. Additional unvaccinated heifers (No. 318, 319, 327, 329, 334, and 335) and 1 unvaccinated mature animal (cow C) were kept with another herd, to be described elsewhere. All but one of this latter group were in the second trimester of gestation at the time the other animals with which they mingled were calving. There was no evidence of brucellosis in the cattle of either control group prior to their exposure. The unvaccinated cattle of the second group were exposed in the same manner as the controls in herd 5 and are considered with them in this paper.

All animals were blood-tested at monthly intervals and more frequently immediately after vaccination. End titers of the serums were determined by means of the conventional tube-agglutination test, using antigen supplied by the BAI.

At each parturition, including those after exposure to a virulent culture in 1950, portions of placentas and quarter samples of colostrum were cultured on thiamine-enriched-tryptose or Albimi-Brucella-agar plates containing 0.0125 mg. of sodium azide per milliliter of medium. When placentas could not be obtained, swabbings of the cervices furnished the inoculum. After the first two parturitions, all cultures were incubated at 37 C. in an atmosphere of 10 per cent CO_2 and examined every other day for a week. The material obtained after exposure in 1950 was incubated aërobically. Suspected colonies were identified by means of known Brucella antiserums.

Portions of placenta as well as liver, lung, spleen, meconium, and stomach contents from aborted fetuses were also cultured, as described previously, and inoculated into guinea pigs which were destroyed and cultured four to five weeks after inoculation.

The agglutinin responses of the three groups from the first through the twenty-fourth months after the first vaccination have been described in the first paper of this series.[2] Following revaccination, there was a rapid rise in agglutinin titer which then fell, on the average, below 1 : 100 within three months. The average titers of the revaccinated groups were between 1 : 25 and 1 : 100 at the time of the first parturition, while the average titers of the animals in group 1 (single-vaccinated animals) were below 1 : 25 after the sixth month following vaccination. The titers in the revaccinated groups continued to decline and levelled off below 1 : 25, on the average, after the twenty-fourth month following the first vaccination.

The previous report[2] also described the course and bacteriological examinations of the first pregnancies, including the details of a spontaneous outbreak of infection in which 2 of the animals in group 1 and 1 from each of the revaccinated groups became infected and were slaughtered.

Fifteen of the animals in group 1 (single-vaccinated animals) were bred for the second pregnancy during 1948-1949. Three of these (No. 2, 26, and 68) failed to conceive. Cow 19 aborted at 143 days. At that time and thereafter, her serum was negative to the agglutination test, at a dilution of 1 : 25. Bacteriological examination of the placenta, fetus, colostrum, and repeated quarter milk samples from this animal failed to reveal Brucella. All other animals in this group delivered living vigorous calves at full term.

Nineteen cows in group 2 (vaccinated at 8 and 14 months) were bred for the second pregnancy. Two of these (No. 12ZC and 62) failed to conceive. Cow 82 delivered a mummified fetus at 172 days. There was no evidence that brucellosis was responsible for this abnormal parturition. All others in this group gave birth to normal calves.

Two (No. 7 and 29) of the 19 animals in group 3 (vaccinated at 20 months) failed to conceive when bred for the second pregnancy. Cow 9ZC aborted in 243 days. Brucella were not isolated from the placenta or fetus on cultures or from the tissues of guinea pigs inoculated with placenta or fetal tissues. However, a strain of *Br. abortus* which did not require added CO_2 for growth was recovered from cultures of the colostrum from one quarter. This strain produced progressive brucellosis when inoculated into guinea pigs, and grew in the presence of concentrations of thionine blue inhibitory for strain 19.[3] Cow 9ZC had failed to conceive when bred for the first pregnancy during 1947 and had maintained a persistent titer of 1 : 800 after her second vaccination. It was concluded that she had probably become infected during the natural outbreak of brucellosis which had occurred in 1947. Repeated milk cultures during the next six months failed to yield Brucella. All other animals in group 3 carried their calves to term.

Six animals (No. 7, 15, 25, 35, 65 and 79) described in the previous report[2] were removed from the herd before exposure because of sterility, mastitis, or other causes.

While breeding for the third pregnancy in herd 5 was in progress in 1949, 6 unvaccinated heifers in the second trimester of pregnancy were used for a titration of the culture to be used for exposure. These animals were housed in an isolation barn on another farm. Three of the heifers were given 1 million organisms and 3, 10 million organisms of strain 2308 by instillation into the conjunctival sac. The animals were sacrificed three months after inoculation, and cultures and guinea pig inoculations made from the placenta, uterine fluids, fetal tissues, and lymph nodes, spleen, and liver of the cows. Brucella were recovered from the lymph nodes of 2 of the 3 animals given 1 million organisms, but there was no uterine invasion in either of these at the time of necropsy. Cultures of the uteri of all 3 of the heifers given 10 million organisms yielded Brucella. On the basis of this titration, it was unanimously decided by the University of Wisconsin Brucellosis Research Committee that it would be advisable to use approximately 10 million organisms for exposure of the experimental herd on Jan. 16, 1950.

The Brucella culture used for exposure was one which had been isolated from the uterus of one of the heifers employed in the titration just described. The saline suspension for inoculation was prepared from a 36-hour culture grown on tryptose agar. Platings before and after inoculation of the cattle gave viable cell counts of 12×10^7 organisms per milliliter. One-tenth milliliter of this suspension was instilled into the right conjunctival sac of each animal. Examination of colonial morphology on replicate platings of this suspension revealed only smooth organisms. Three of 4 guinea pigs inoculated intraperitoneally with 120 organisms from the cattle challenge suspension were found to be infected when destroyed four weeks after inoculation, as were 4 of 4 guinea pigs in each of two lots given 1.2×10^6 and 1.2×10^4, respectively.

Of the 22 controls (table 1) which were exposed to infection, 4 were not pregnant and the others were between twenty-eight and 163 days in gestation, with a mean of 111 days at the time of exposure. The range of time in gestation at exposure in group 1 (table 2) was from eighty-three to 163 days, the mean was 131 days. In group 2 (table 3), 1 was nonpregnant and the other 17 were in the eighty-fourth to 157th days of pregnancy, with an average of 118 days. The 16 cows in group 3 (table 4) ranged from forty-nine to 163 days in pregnancy and averaged 128 days.

After exposure, agglutination tests were made at approximately ten-day intervals. At parturition of each animal, cultures and guinea pig inoculations were made as described previously. Repeated attempts to isolate Brucella were made from quarter milk samples from most of the animals, and especially from those which failed to yield the organism at parturition but had suspicious or positive serum—or whey—agglutination reactions. Twenty animals were bred after the postexposure parturition and killed at intervals when most of them were in advanced pregnancy. Cultures and

TABLE 1—The Response of Unvaccinated Control Cows to Brucella Exposure

Cow (No.)	Titer at exposure	Days service to exposure	Days exposure to parturition	Duration pregnancy (days)	Fate of calf L or D*	Brucella recovery: Placenta or fetus	Brucella recovery: Colostrum	Brucella recovery: Milk	Maximum** titer post-exposure
C	—†	134	84	218	D	+	+	1/4‡	12,800
318	—	79	142	221	D	+	—	0§	12,800
319	—	120	66	186	D	+	—	0/3	1,600
327	—	Nonpregnant	...	...	...	..	..	1/1	6,400
329	—	Nonpregnant	...	...	...	..	..	1/1	25,600
334	—	82	194	276	L	—	+	1/2	12,800
335	—	144	72	216	D	+	—	0/5	100
453	—	85	170	255	L-W	—	—	0/9	1,600
455	—	Nonpregnant	...	...	...	..	..	0	100
461	—	29	229	258	L-W	—	+	2/3	3,200
526	—	115	60	175	D	+	—	0/4	12,800
528	—	113	52	165	D	+	—	1/5	12,800
535	—	28	248	276	L	—	+	0	12,800
675	—	150	61	211	D	+	—	0/4	12,800
676	—	107	169	276	L	—	+	0	3,200
677	—	Nonpregnant	...	...	...	..	..	0	200
683	—	163	37	200	D	+	—	0/7	1,600
689	—	132	106	238	L-W	+	—	0	3,200
692	—	115	90	205	D	+	—	0/4	12,800
693	—	124	66	190	D	+	+	1/4	12,800
695	—	161	52	213	D	+	—	1/5	6,400
696	—	124	68	192	D	+	+	1/6	800
	Mean	111	99	220	Total	13/18	7/18	10/67	

*L = calf living; L-W = calf living but weak; D = calf dead. **Highest dilution of serum with complete or incomplete agglutination. †— = negative at 1:25 dilution. ‡Number of times Brucella recovered/number of times quarter samples cultured. §0 =no cultures made.

guinea pig inoculations were made from uterine contents and various lymph nodes of these cattle. Included among these animals were a number which gave serological or other evidence of infection but from which it had not been possible to isolate Brucella at parturition or from repeated milk samples.

EXPERIMENTAL RESULTS

Controls.—As may be seen in table 1, 12 of the 18 unvaccinated pregnant control cattle which were exposed to infection aborted, 3 gave birth to premature weak calves, and 3 delivered normal vigorous calves at full term. The mean duration of pregnancy in this group was 220 days. *Brucella abortus* was recovered from all but one of these animals (No. 453). This cow developed a maximum agglutinin titer of partial at a dilution of 1:1600, and her serum continued to react at a dilution of 1 : 100 or higher through the 275th day postexposure. Brucella could not be isolated from her placenta, colostrum, uterine swabbings, nine different quarter milk cultures, repeated cultures of fluid aspirated from bilateral carpal hygromata, or from tissues taken at necropsy.

Of the 4 controls which were nonpregnant at exposure, 2 (No. 327 and 329) were lactating from the first pregnancy. Both of these developed positive agglutination reactions and *Br. abortus* was recovered from the milk. Cow 455 developed only a suspicious agglutinin titer, and cow 677 a transitory positive serological reaction. Cultures and guinea pig inoculations of tissues taken at autopsy from these 2 cows did not yield Brucella.

The maximum agglutination titers of each of the control animals are given in table 1.* The incubation period for the development of titers of 1 : 100 or higher varied from sixteen to eighty days after infection, with a mean of thirty-three days.

Group 1.—Of the 14 cows which had been vaccinated once, 3 aborted (No. 2, 21, and 72) and 1 (No. 16) had a weak calf after 247 days' gestation. It may be seen in table 2 that infection was not demonstrable in cow 2 and that her serum did not react in the agglutination test at dilutions greater than 1 : 25. The other 3 animals (No. 16, 21, and 72) with abnormal parturitions were demonstrated to be infected. All other animals in the group had normal calves at full term.

Cow 31, which had had a normal calf, developed an agglutination titer of 1 : 100 at fifty-six days after exposure and continued to react above that level for the next year, with a peak titer of 1 : 800 at 310 days. Brucella were not recovered from any of 15 successive quarter milk samples or from tissues obtained at necropsy. Several other animals in the group had transient and fluctuating agglutination reactions but infection could not be demonstrated in any of these.

*The maximum agglutination titers observed during the postexposure period are given with the parturition data for each of the experimental animals, (Tables 1 to 4). Since the agglutination titers of many of the vaccinated animals fluctuated during the observation period, this might give a misleading impression of the distribution of reactor and suspect animals in the various groups. The complete agglutination test records have been prepared in tabular form and mimeographed copies will be furnished on request to the authors.

TABLE 2—The Response of Cows, Vaccinated at 8 Months of Age (Group 1), to Brucella Exposure*

Cow (No.)	Titer at exposure	Days service to exposure	Days exposure to parturition	Duration pregnancy (days)	Fate of calf L or D	Brucella recovery: Placenta or fetus	Brucella recovery: Colostrum	Brucella recovery: Milk	Maximum** titer postexposure
2	—	145	78	223	D	—	—	0/7	25
11	25	112	167	279	L	—	—	0	100
16	—	139	108	247	L-W	+	+	0/1	3,200
19	—	150	128	278	L	—	—	0	50
21	—	131	25	156	D	+	+	0/3	50
26	—	112	170	282	L	—	—	0	100
31	—	158	126	284	L	—	—	0/15	800
33GC	—	163	123	286	L	—	—	0	50
11ZC	—	100	182	282	L	—	—	0/1	200
63	—	144	130	274	L	—	—	0	50
68	—	139	143	282	L	—	—	0	50
70	—	137	143	280	L	—	—	0/10	200
72	—	83	102	185	D	+	+	1/1	3,200
75	—	120	152	272	.	—	---	0/4	100
	Mean	131	127	265	Total	3/14	3/14	1/42	

*For abbreviations see table 1.

Group 2.—The details of the responses of the animals in group 2 are presented in table 3. Two of the 17 pregnant animals (No. 20ZC and 71) in this group aborted. Cow 20ZC aborted twin fetuses on the 210th day of gestation, ninety-seven days after exposure. At that time, she had a serum agglutinin titer of 1 : 50, which was the maximum attained. Brucella were not recovered from materials taken at parturition, six weekly quarter milk samples, or tissues obtained at necropsy. It was concluded that the abortion was not associated with brucellosis. *Brucella abortus* was recovered from the aborted fetus from cow 71.

Four cows (No. 12, 32, 12ZC, and 66) which delivered viable full-term calves were demonstrated to have mammary gland infection, although Brucella were not recovered from placentas or uterine swabbings. Each of these animals had high titers of serum agglutinins.

Cultures of the bronchial lymph nodes of cow 74 yielded *Br. abortus* eighteen months after exposure. This animal had had a normal calf and Brucella were not isolated

TABLE 3—The Response of Cows, Vaccinated at 8 and 14 Months of Age (Group 2), to Brucella exposure*

Cow (No.)	Titer at exposure	Days service to exposure	Days exposure to parturition	Duration pregnancy (days)	Fate of calf L or D	Brucella recovery: Placenta or fetus	Brucella recovery: Colostrum	Brucella recovery: Milk	Maximum* titer post-exposure
3	—	139	140	279	L	—	0	0**	200
6	—	89	193	282	L	—	—	0	50
12	—	145	133	278	L	—	—	1/1	12,800
22	—	105	178	283	L	—	—	0	50
23	—	114	174	288	L	—	—	0/6	100
30	—	86	203	289	L	—	—	0/1	100
32	—	133	155	288	L	—	+	0	6,400
12ZC	P 50†	58	227	285	L	—	—	2/4	12,800
11GC	—	64	219	283	L	—	—	0	50
20ZC	—	113	97	210	D¶	—	—	0/6	50
62	—	146	132	278	L	—	—	0/1	50
66	P 50	155	137	292	L	—	—	2/6	3,200
67	—	157	125	282	L	—	—	0	25
71	—	152	46	198	D	+	—	0/7	12,800
73	P 25	137	146	283	L	—	—	0/14	200
74‡	P 25	84	203	287	L	—	—	0/1	100
80	—	135	155	290	L	—	0	0**	50
82	—	Nonpregnant	...	...	...	..	..	0	50
	Mean	118	156	276	Totals	1-17	1/17	5/47	

*For abbreviations see table 1. **Nonfunctional mammary gland. †P 50 = partial agglutination at a dilution of 1 : 50. ‡See text for details of isolation of organisms at necropsy. ¶Twins.

TABLE 4—The Response of Cows, Vaccinated at 8 and 20 Months of Age (Group 3), to Brucella Exposure*

Cow (No.)	Titer at exposure	Days service to exposure	Days exposure to parturition	Duration pregnancy (days)	Fate of calf L or D	Brucella recovery: Placenta or fetus	Brucella recovery: Colostrum	Brucella recovery: Milk	Maximum** titer post-exposure
1	25	150	125	275	L	—	—	0/10	100
4	P 25	83	159	242	D	+	0	0**	12,800
10	—	122	79	201	D	+	—	0/2	12,800
18	—	163	122	285	L	—	—	0	50
24	—	49	236	285	L	—	—	0/1	100
28	—	50	236	286	L	—	—	0	50
29	25	125	155	280	L	—	—	0/1	100
9ZC†	400	157	128	285	L	—	—	1/2	12,800
1FC	P 50	108	179	287	L	—	—	0/7	100
35ZC	P 25	134	146	280	L	—	—	0/12	800
69	—	147	65	212	D	+	—	0/3	12,800
76	P 50	146	136	282	L	—	—	0/3	100
78	P 25	149	132	281	L	—	—	0	50
83	—	157	124	281	L	—	0	0*	50
85	P 25	158	121	279	L	—	—	0/1	100
86	—	146	135	281	L	—	—	0/1	100
	Mean	128	142	270	Totals	3/16	0/16	1/43	

*For abbreviations see table 1. **Nonfunctional mammary gland. †See text.

at parturition or from the milk. Her agglutinin titer reached a maximum of 1 : 100 and fluctuated between 1 : 100 and 1 : 50 during the entire postexposure period.

Cow 82, which was nonpregnant, exhibited no signs of infection. All other animals in the group had normal parturitions and only fluctuating suspicious agglutination reactions.

Group 3.—Three of the 16 cows (No. 4, 10, and 69) in group 3 aborted and *Br. abortus* was recovered in each case, as is shown in table 4. All other animals in the group produced vigorous calves at full term.

Cow 9ZC, whose previous infection is described in the section on Materials and Methods, was shown to have mammary gland infection.

Cow 35ZC had a serum agglutinin titer of 1 : 100 thirty-five days after infection, and thereafter reached a maximum titer of 1 : 800 at 118 days postexposure, with definite fluctuations. Repeated milk cultures as well as the bacteriological examinations at parturition and at necropsy failed to demonstrate infection.

The results of the exposure are summarized in table 5.

TABLE 5—Results of Exposure

	Infected	Noninfected	Total
Controls	19	3	22
Group 1	3	11	14
Group 2	5	13	18
Group 3	4	12	16

DISCUSSION

It is clear from the data presented that, in comparison with the unvaccinated control animals, all of the vaccinated groups had a considerable degree of resistance to induced infection. This is reflected both in the abortion and infection rates. The only manifest difference in the response of animals vaccinated once from that of those which were revaccinated was the greater tendency of the revaccinated animals to develop suspicious or positive agglutination reactions in the absence of demonstrable infection. This introduces a difficulty in classifying the response of such animals to the induced infection. In this paper, failure of recovery of Brucella from a particular animal is arbitrarily considered as an indication of resistance. However, despite the repeated bacteriological examinations made of animals with positive agglutination reactions, it can not be stated absolutely that they were not infected. In terms of the criteria necessarily used for the classification of animals in the control of bovine brucellosis in the field, this tendency on the part of revaccinated animals to develop agglutinins following exposure, as well as their lack of manifestly increased resistance, are factors which would militate against revaccination as employed in this experiment in the field.

The rates of abortion and infection observed in the present trial, in animals vaccinated as calves and exposed during the third pregnancy, are of the same order as those described for vaccinated animals challenged during the first gestation period, as given in a review of such tests.[1] This resistance to infection of the cattle in group 1 differs markedly from that of calfhood-vaccinated animals infected during the third pregnancy in a previous experiment from this institution.[4] Of the 23 animals exposed in that test, 12 aborted and 2 had premature weak calves in contrast to the 14 animals of group 1 in the present experiment where 3 aborted and 1 had a premature weak calf. Similarly, 18 of the 23 cows in the earlier experiment were demonstrated to be infected, whereas only 3 of the 14 animals of the latter herd (No. 5) were shown to be carriers. Interpretation of these discrepant results is difficult from the limited data available. Birch *et al.*[3] have described a decline, with the passage of time, in the resistance of vaccinated cattle naturally exposed to Brucella which is consistent with the lack of protection observed in our previous experiment.[4] On the other hand, workers* at the Beltsville, Md., Animal Disease Station[10] found calfhood-vaccinated cattle exposed by the conjunctival route during the fourth and fifth pregnancies to be highly resistant to infection.

Although the same strain of *Br. abortus*

*Since this paper was accepted for publication, the report of experiments at the Beltsville Station on the duration of resistance engendered by vaccination with strain 19 has appeared (Manthei, Mingle, and Carter: Proc. 88th Annual Meeting AVMA, *126*, 1951). In their study, calfhood-vaccinated animals were exposed in the third pregnancy with 100 x 10^6 organisms of strain 2308. The proportion which became infected was not significantly different from that observed in the comparable group (group 1) exposed with 12 x 10^6 organisms of the same strain in the present investigation.

(2308) was used for conjunctival exposure in the trials in our laboratory and at the Beltsville Station, the dosage varied. In our earlier trial, the inoculum was 33 x 10^6 organisms, while less than half that number (12 x 10^6) were administered in the present experiment. The numbers used by the Beltsville workers have not yet been published. One hypothesis which has been advanced is that there is a critical number of organisms given as a challenge inoculum above which the resistance of a considerable proportion of vaccinated animals is overwhelmed, while numbers considerably below that level will still infect unvaccinated cattle.

Manthei[11] has presented the following data which appear to support this contention. All of 12 unvaccinated cattle exposed by the conjunctival route with 26.1 x 10^6 organisms of strain 2308 became infected, as did 10 of 15 heifers vaccinated as yearlings and similarly exposed during the first pregnancy. An inoculum of 15.6 x 10^6 organisms infected all of 18 controls and 22 of 34 vaccinated animals. Inoculums of 7.4 x 10^5 and 3.7 x 10^5 organisms infected 8 of 9 and 7 of 9 controls and 2 of 9 and none of 8 vaccinated animals, respectively. Analysis of the difference in the response of vaccinated animals given 15.6 x 10^6 and 7.4 x 10^5 organisms, respectively, shows it to be significant ($X^2 = 5.2$, $p < 0.05 > 0.01$). Although the difference between the reactions of the two groups of animals given 15.6 x 10^6 and 3.7 x 10^5 organisms, respectively, is highly significant ($X^2 = 10.87$, $p < 0.01$), it would be dangerous to extrapolate from these small numbers, particularly in view of the resistance exhibited by vaccinated animals exposed to more than 10^6 organisms in other trials.[1]

Further data on the influence of the number of organisms on the response of vaccinated animals may be obtained from several experiments of British workers,[12-15] in which two different challenge doses of another strain (544) were employed. Grouping the results of several experiments, it may be seen that conjunctival exposure with 15 x 10^6 organisms resulted in infection of all of 9 controls but of none of 9 adult-vaccinated heifers. All but 1 of 42 controls exposed with 150 x 10^6 organisms became infected, as did 8 of 48 vaccinated animals. Again, very few animals were given the smaller inoculum, but the difference in response of the vaccinated animals in the two groups is not significant ($X^2 = 1.1$, $p > 0.20$).

It is clear that experiments involving numbers of animals adequate for statistical analysis are necessary to establish the significance of the size of the inoculum in vaccination experiments. In any event, the approximately threefold decrease in numbers of organisms involved in the two trials of this laboratory do not approximate the almost fifty-fold change required for differences to be manifested in Manthei's investigation. Should further data become available which might indicate the critical nature of the exposure dose, it would be of interest to determine whether revaccination would protect animals against numbers of organisms which would overwhelm the resistance of animals vaccinated only once.

Another possible source of the differences in response of singly vaccinated cattle in our two studies is the possible natural exposure to virulent infection which some of the animals of herd 5 had during their first pregnancy. It might be postulated that such an exposure served to increase the resistance already engendered by vaccination. This would make difficult interpretation of the observation of Birch *et al.*[3] that resistance of vaccinated animals declined despite repeated natural exposures. Furthermore, there was no evidence of exposure in the present herd, such as increases in agglutinin titer before experimental exposure.

The relatively rapid loss of agglutinins following revaccination seen in this and another larger group of animals[8] might be attributable to a rapid elimination from the body of the organisms contained in the second dose of *Br. abortus* strain 19, as a result of resistance engendered by the first vaccination. The authors have previously discussed the similarity of this response to the so-called "Koch reaction" in tuberculosis, and a similar response has been described in Brucella reinfection experiments in guinea pigs.[16, 17] In cattle, this clearance mechanism apparently prevents the establishment of the second inoculum of strain 19 in the tissues. This refractory state, as indicated by the agglutinin response, has been observed in some animals which were revaccinated as long as five years after calfhood vaccination.[8] Whether any increment of resistance might be ex-

pected from repeated vaccination, in view of the apparently transitory contact between the agent and the host tissues, appears problematical. It would be of interest to determine the effect of inoculation, at different times after calfhood vaccination, of soluble antigens (with adjuvants) prepared by various methods from strains of high pathogenicity (cf. [18]). Such experiments as well as tests of other new vaccines would require the development of exposure methods capable of overcoming the resistance produced by calfhood vaccination with strain 19 at a predictable and reproducible rate.

SUMMARY

Three groups of Holstein-Friesian heifer calves were vaccinated with *Brucella abortus* strain 19 at 8 months of age. Two of these groups were revaccinated at 14 and 20 months of age, respectively. In the third pregnancy, the cows of these groups, along with unvaccinated controls, were exposed by the conjunctival route with 12 x 10^6 organisms of *Br. abortus* strain 2308.

Fifteen of the 18 pregnant controls had abnormal parturitions, with Brucella infection demonstrable in 17. Two of 4 nonpregnant controls were also infected.

Of the 14 cows vaccinated once, 4 had abnormal parturitions and Brucella infection could be shown in 3 animals.

Of the 18 cows vaccinated at 8 and 14 months of age, 2 aborted and 5 were found to be infected.

Three of 16 animals vaccinated at 8 and 20 months aborted, and 4 were found to be infected.

An increment in resistance engendered by revaccination at either 14 or 20 months of age with *Br. abortus* strain 19 was not apparent under the conditions of this experiment.

The immunological significance of the results and the significance of the severity of challenge in vaccination experiments are discussed.

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Histochemical Methods in the Study of Telangiectasis

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AN EXPLANATION FOR the occurrence of telangiectasis in the bovine liver is widely sought because of financial losses to cattlemen and packers alike. The extent of these losses has been well described in a previous report.[1] Therefore, this paper will be concerned with the pathogenesis of telangiectasis.

OCCURRENCE

"Telang" lesions are widely distributed in the bovine species. Occasionally, cattle direct from the range can be found to have the lesion. It may occur in older dairy cattle, in which lesions can be quite extensive. Cattle used for work purposes in Europe may also develop the condition. By far, however, the largest group affected with telangiectasis is the feedlot beef animal. Slaughterhouse records show that telangiectasis is only responsible for a small percentage of livers condemned. However, if we consider microscopic as well as macroscopic lesions and its presence with other pathological entities, the condition is more widely spread than is generally appreciated. Livers condemned because of abscesses often show telangiectasis, but distomiasis-condemned livers are seemingly less involved. Livers that have passed meat inspection may have a few lesions occasionally.

Data from previous reports indicate that telangiectasis is occurring to the largest extent early in the feedlot. Jensen *et al.*[2] reported that telangiectasis largely occurred between the fortieth and eightieth day in the feedlot and was followed by the highest incidence of abscess formation. It is of interest to note from this report that the concentrates each animal consumes during the fattening period follow a definite pattern. This pattern indicates that the amount of feed ingested per animal reaches a maximum on the sixty-seventh day and is followed by a sudden drop in feed intake. This is suggested to result from abscess formation. During the remaining fattening period, the quantity of feed consumed shows a gradual increase but never reaches the previous maximum. Robinson *et al.*[3] stated that 81 per cent of beef livers were affected with telangiectasis at the fifty-sixth day in the feedlot, and that at 160 days in the feedlot only 2.4 per cent were affected.

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Microphotography by Lorne Hardaker, School of Veterinary Medicine, University of California.

PATHOGENESIS OF TELANGIECTASIS*

Correlation of available information describing the pathogenesis of telangiectasis in the bovine liver suggests the formation of enlarged blood spaces due to necrosis of the hepatic cell. The cause of necrosis is not offered. Many questions arise from such an explanation, because the average telang lesion prepared by routine histological techniques shows the surrounding parenchyma as normal with only a few leukocytes present. Figure 1 illustrates such a lesion with blood spaces of various sizes occupying a portion of one hepatic lobule. An inspection of this lesion reveals the coalescing of smaller blood spaces to form larger or cavernous spaces. The surrounding unaffected parenchyma shows little alteration; since even the endothelium lining the various-sized blood spaces is largely intact. Blood present in these spaces has varying amounts of erythrocytes and numerous hepatic cell nuclei—all of which indicate a blood stasis. This is evident grossly by the dark red appearance.

Studies of numerous sections with the use of various histological techniques offer some elucidative information as to how telangiectasis develops. Microscopic or pinpoint lesions reveal that the condition develops primarily as the result of an intralobular defect. The loss of hepatic cells from the cords is followed by mechanical distortion caused by an increased blood volume in the damaged site. The application of connective tissue stains to telang lesions reveals the reticular stroma alterations. Figure 2, stained for reticulum, shows hepatic cords at the periphery of a small telang lesion. The delicate reticular stroma of the hepatic cells and sinusoids has been

*All figures reported in this paper have been taken from colored microphotographs.

greatly distorted, with the parenchymal cells being washed away from their normal anastomotic cords. The distorted reticulum often appears condensed along the broken hepatic cord (fig. 2, B). Blood flowing through the enlarged spaces contains numerous hepatic nuclei and fragments of reticulum. Cytological studies of hepatic cells not involved with pressure or dislocation do not show any significant alterations. Mitochondrial stains[4] applied to such cells show these mitochondrial structures to be active cellular constituents. This coincides with the studies made by Getty.[5]

Histochemical techniques demonstrating various metabolic substances indicate that telangiectasis occurs largely in those livers having a high glycogen storage. Best's carmine to demonstrate glycogen and Sudan III to demonstrate fats reveal that telangiectatic lesions occur most commonly in those lobules having higher glycogen accumulation and less fats. Figure 3 demonstrates a pin-point lesion stained for polysaccharides by the Hotchkiss technique.[6] This technique stains both glycogen and the more complex polysaccharides. To distinguish the complex polysaccharides from glycogen, it is necessary to hydrolyze glycogen by saliva in a control section; this leaves only the faint outline of the hepatic stroma. In fig. 3, the hepatic cells stain darker because their polysaccharides are mostly glycogen concentration. In this figure, the hepatic cords at the periphery of the telang lesion are concentric in arrangement, which indicates they have been pushed by the blood laterally and in the direction of blood flow toward the central vein. Those hepatic cords surrounding the lesion are

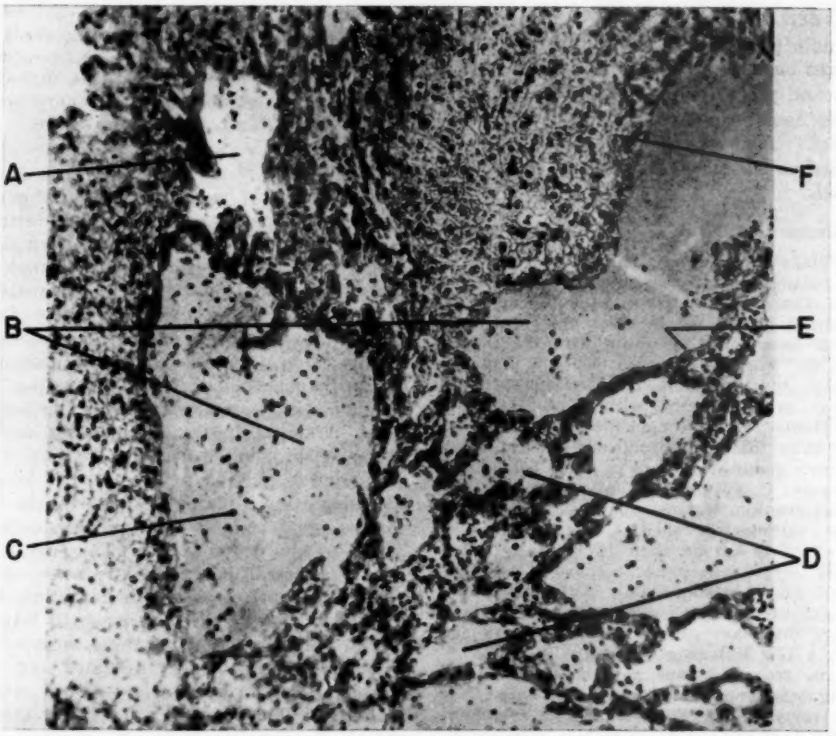

Fig. 1—Telangiectatic lesion; bovine liver. (A) central vein, (B) cavernous blood spaces, (C) hepatic nuclei in blood spaces, (D) small blood spaces, (E) endothelium lining blood space, (F) normal surrounding parenchyma. Bouin's fixative; hematoxylin and eosin stain. x 100.

heavily laden with polysaccharides, while the adjacent lobule (E) contains less polysaccharides.

Glycogen is stored intracellularly from the central region of the lobule toward the periphery and is released in the reverse order.[7] Before the hepatic lobule becomes filled with glycogen, those hepatic cells immediately surrounding the portal canal store glycogen. These peripheral hepatic cells stain darker than the other parenchymal cells of the lobule after Bouin's fixation and routine stains. This staining reaction suggests that glycogen may be stored in a more complex form in the peripheral cells of the lobule, which may be related to the close association of the mast cells found normally in the connective tissue of the portal canal. That most telang lesions occur at the periphery of the lobule has been demonstrated by Julian with the use of vinylite casts.[8] However, the telang lesion does not appear to involve the most peripheral hepatic cells of the lobule that are directly in apposition to the connective tissue of the portal canal.

The application of a suitable counterstain after the Hotchkiss technique for polysaccharides shows the structural details of the hepatic cord in relation to the metabolic accumulations. In such preparations, glycogen is occasionally found between the hepatic cell and the endothelium. This extracellular accumulation of glycogen is shown in fig. 4 at the entrance of the hepatic sinusoids. The large globular accumulation of glycogen shows endothelial distortion with the adjacent cell having a few erythrocytes under the endothelium. The extracellular glycogen accumulation is hydrolyzed by salivary digestion on the control sections. Details of this accumulation will

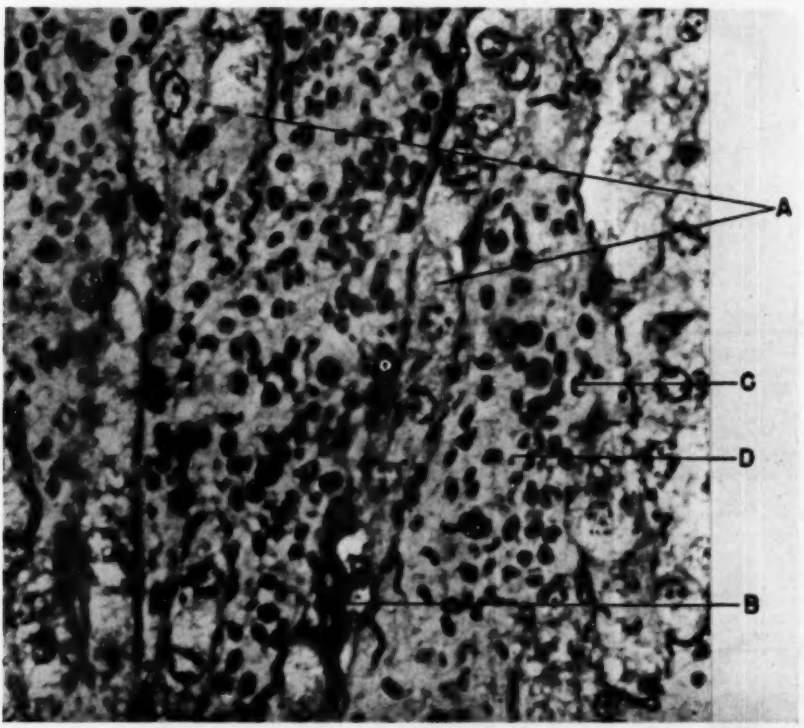

Fig. 2—Periphery of a small "telang" lesion. (A) hepatic cords, (B) distorted and condensed reticulum along hepatic cord, (C) reticulum fragment in blood space, (D) blood space containing erythrocytes and hepatic nuclei. Zenker-formol; nitrocellulose; modified Foot and Menard method for reticulum and hematoxylin; 6μ. × 1,000.

be discussed in relationship to hepatic cord structure.

HISTOLOGICAL STRUCTURE OF THE HEPATIC CORD IN RELATION TO EXTRACELLULAR GLYCOGEN ACCUMULATION

Blood flowing through the hepatic lobule takes a tortuous course in the sinusoids. This arrangement is due to anastomoses at intervals of from three to eight hepatic cells along the cord. Preparations in which the sinusoids are dilated bring out this arrangement.

The detailed structure of the hepatic cord reveals that the exchange of metabolites from the sinusoids passes through an endothelial layer, intercellular matrix, and cellular membrane before entering the cytoplasm of the hepatic cell. The release of metabolites from the hepatic cell occurs through the passage of the same structures. The intercellular matrix between the hepatic cell and endothelium forms the space of Dissé. This area is readily distorted by the use of heat with paraffin or cold with freezing techniques. Infiltrating fixed specimens with nitrocellulose at room temperature was found to be the most satisfactory method to avoid intercellular artifacts.

Rapid fixation with alcohol-formol and gradual embedding with nitrocellulose followed by slow evaporation until the blocks have the consistency of hardened jello allow thin sectioning. Sections 2 to 4 μ thick, are affixed to slides directly from the knife. Staining with Hotchkiss technique for polysaccharides and Pollak's rapid trichrome[9] as counterstain gives accurate cellular details in relation to polysaccharides. Such a preparation is represented by fig. 5. This microphotograph is rather unique in that several phases of telangiectatic pathology can be observed. The extracellular globular mass of glycogen (B) is shown between the hepatic cell (A) and endothelial nucleus (C). This misplaced accumulated gly-

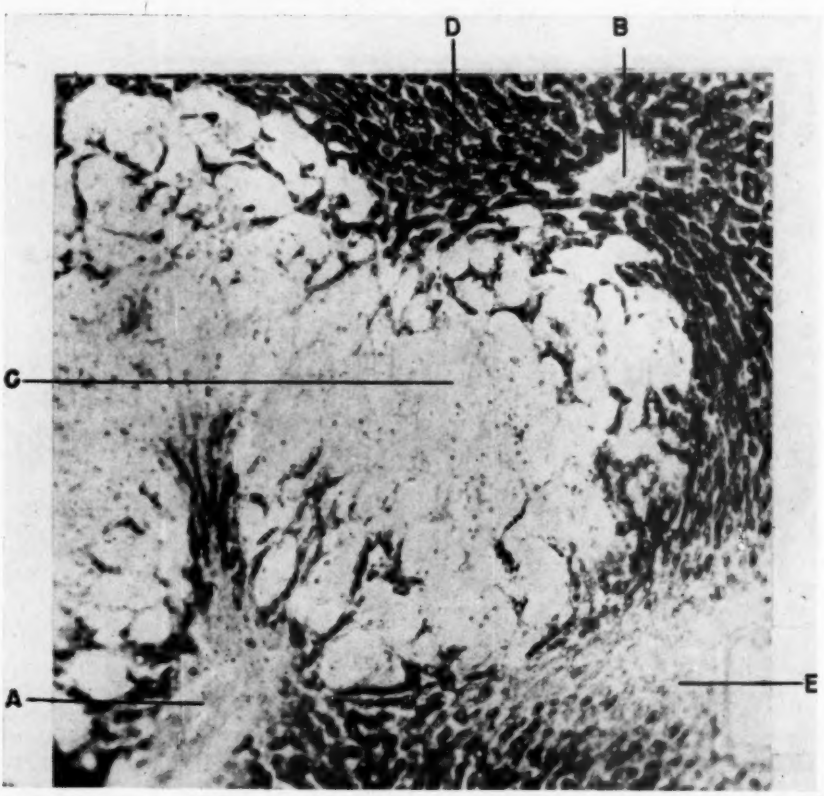

Fig. 3—Pin-point lesion stained for polysaccharides. (A) portal canal, (B) central vein, (C) blood space, (D) hepatic cords, (E) central region of adjacent lobule. Carnoy fixative; Hotchkiss technique for polysaccharides. x 100.

cogen takes the shape of the lining endothelium and hepatic cell with the intercellular substance preventing its spread. These glycogen granules vary in size from about 1 μ in diameter to ultramicroscopic, and some stain as glycogen particulate granules. It also appears considerably more diluted than the intracellular glycogen. The hepatic cell (A) does not show any cellular membrane disruption, and the outline of the extracellular glycogen appears to be due to the condensation of the intercellular substance. As this mass of extracellular glycogen accumulates, the endothelium becomes distorted and eventually ruptures, releasing glycogen granules into the sinusoids. That portion of glycogen released into the sinusoids as glycogen particulate granules can often be seen in the blood spaces as pleomorphic coccoid bodies. These coccoid bodies appear to have a homogeneous polysaccharide content. Rupturing of the endothelium may follow, with erythrocyte accumulation in the space occupied by extracellular glycogen. The presence of blood in the intercellular matrix causes dislodgment of the hepatic cell with the eventual erosion of the cell. Area K in fig. 5 illustrates blood flowing through a break in the cord where the hepatic cell is found in the sinusoid as a cytoplasmic mass (H) with an extruded nucleus (O). The loss of more than one hepatic cell between an anastomotic cord results in the displacement of a group or island of hepatic cells. Occasionally, free entire hepatic cells are seen in the blood spaces of a telangiectatic lesion. Rupture of the endothelium from the release of extracellular glycogen may follow with the process of healing, if blood does not accumulate in the site of damage to cause hepatic cell erosion. Such an area is represented in fig. 5 by thickening of the intercellular matrix (D) leaving a clear space filled with fluid (E). The hepatic cell (N) adjacent to the repair site is in telophase, indicating this cell has undergone cellular

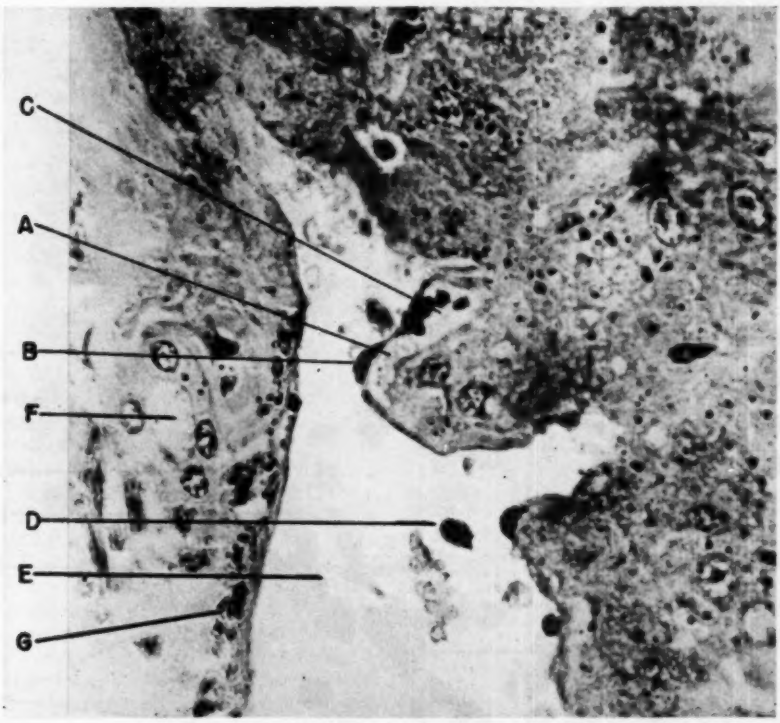

Fig. 4—Portal vein determination into hepatic sinusoid. (A) extracellular accumulation of glycogen, (B) endothelium, (C) eythrocytes under distorted endothelium, (D) eroded pyknotic nucleus, (E) portal vein termination into sinusoid, (F) bile duct in portal canal, (G) accumulated polysaccharide staining granules in connective tissue of portal canal (mast cell granules). Carnoy fixative; nitrocellulose; Hotchkiss technique for polysaccharides and Pollak rapid trichrome stain; 4 μ, x 1,000.

activity, a period of rest, and is in a state of cellular division. The hepatic cell (F) in the same cord is also in cellular division.

finding of the telang lesion in close association with the portal vein.

Hepatic cords separating the blood spaces

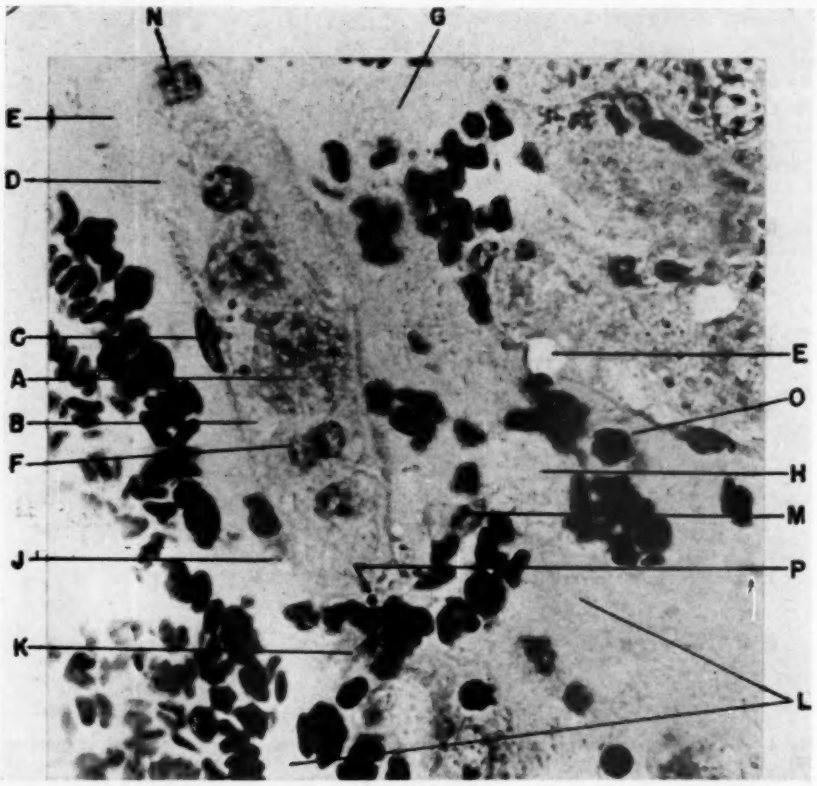

Fig. 5—Hepatic cord at the periphery of a small "telang" lesion. (A) hepatic cell, (B) extracellular accumulation of glycogen, (C) endothelial nucleus, (D) thickening of intercellular area, (E) clear space following release of glycogen, (F) hepatic cell in telophase, (G) cytoplasm of hepatic cell in blood space, (H) cytoplasm of eroded hepatic cell, (J) damaged endothelial cell, (K) erythrocytes passing between blood spaces, (L) blood spaces, (M) nucleus of disrupted endothelium, (N) hepatic cell in metaphase, (O) extruded nucleus, (P) glycogen particulate granule. Alcohol-formol fixation; nitrocellulose; Hotchkiss technique for polysaccharides and Pollak rapid trichrome stain; 2 μ. x 2,000.

Erosion of the hepatic cell results in an abnormal intersinusoidal flow of blood. The loss of more than one hepatic cell, resulting in a break of the cord between the anastomoses, allows a more direct flow of blood rather than the normal tortuous course in the sinusoids. This results in an increased volume of blood flowing toward the central vein in a portion of the lobule and causing mechanical alteration. The resulting blood space enlarges until the blood pressure equals that of the normal surrounding parenchyma. The periphery of the lobule having a higher blood pressure than the central area might, in part, explain the common

further undergo erosion as a result of blood stasis, causing endothelial damage due to anoxia. This results in the formation of cavernous blood spaces. Giemsa stain applied to sections readily demonstrates the imbibition by the endothelium of pigment granules and the loss of the cytoplasmic outline of these cells in those cords separating the blood spaces.

Larger telang lesions quite consistently show the presence of mast cells. These cells are found in or on the hepatic cords separating the blood spaces and can often be found in clumps. Thionine or toluidine blue readily demonstrate their granule reaction as charac-

teristic of normal and active cells. Their apparent migration in the telang lesion is suggestive of an anticoagulant activity.

FATE OF THE TELANG LESION

The fact that many more telang lesions develop early in the feedlot than are found at slaughter creates an interesting question. What happens to telang lesions after they develop? Several possible explanations might be given. Some lesions may remain as such for a considerable time in the absence of pathogens and with the presence of mast cells preventing blood coagulation. Occasionally in normal sections, an area of a lobule is encountered whose shape and size would indicate that of a small telang lesion which has healed by hepatic cell proliferation. The apparently regenerated area appears as a circular region in the lobule where the hepatic cord arrangement is not in conformity with the other portions of that lobule. Telangiectatic lesions do not show leukocytic invasion. Macerated telang tissue injected intravenously and subcutaneously into rabbits fails to produce any pathological condition in these animals. The use of Machiavello,[10] Gram Weigert,[11] and routine bacteriological stains on sections and smears fail to demonstrate the presence of any organism. Lesions found with a leukocytic invasion indicate a focal hepatitis and are distinguished from telangiectatic lesions. The presence in telang lesions of blood stasis, anoxia, metabolites, and relatively few leukocytes suggests a lesion that is highly vulnerable to bacterial invasion.

CONCLUSIONS

1) Telangiectasis develops from the erosion of hepatic cells, and not from cellular necrosis.

2) Erosion of the hepatic cell occurs from rupture of the endothelium lining the hepatic cord due to the extracellular accumulation of glycogen.

3) Glycogen may be accumulating in the space of Dissé as the result of a defective glycolytic activity in the dynamic state of glycogen metabolism by the hepatic cell.

4) Hepatic cellular erosion explains the presence of numerous hepatic nuclei, stromal and cytoplasmic elements, and the relatively few leukocytes found in the blood spaces forming the telang lesion.

5) Hepatic cell erosion causes increased blood volume in a portion of the lobule resulting in mechanical distortion of the hepatic cords.

6) The release of glycogen and the higher blood pressure at the periphery of the lobule may explain the common finding of telangiectasis in close association with branches of the portal vein.

7) Hepatic cords separating the blood spaces further undergo degeneration due to endothelial damage from anoxia to form cavernous blood spaces.

8) The presence of mast cells in larger lesions suggests an anticoagulant activity of these cells.

9) Small telang lesions may heal by hepatic cell proliferation.

10) Telangiectasis having a blood stasis, anoxia, cellular elements, and few leukocytes suggests a lesion highly vulnerable to bacterial invasion.

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Pathological Aspects of Canine Brucellosis Following Oral Exposure

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THE HISTOPATHOLOGY of *Brucella abortus* infection in dogs has not received the attention which has been accorded infections due to *Brucella suis* in this species. The bacteriological aspects, however, have been considered extensively by a number of workers. The purpose of this paper is to report the pathological changes observed in dogs which were fed aborted material or milk from cattle infected with *Br. abortus* U. S. Department of Agriculture strain 2308.

LITERATURE

Cardona[1] inoculated dogs both intravenously and intraperitoneally with suspensions of *Br. abortus*. Two of the animals had enlarged spleens. The significance of this finding was minimized.

Thomsen[7] observed multiple, miliary, yellowish nodules under the kidney capsule in 2 dogs infected experimentally with *Br. abortus*.

Van der Hoeden[6] reported that no lesions were found in the kidneys of infected dogs. The lymph nodes were not specifically altered. Small foci of proliferating stellate and adventitial cells in the liver were described. Similar changes were present at the periphery of the splenic corpuscles. Many cells in this organ contained pigment. In general, a proliferation of the reticuloendothelial cells was the most noteworthy pathological feature. Van der Hoeden, however, considered that these changes were not specific.

Feldman and associates[2] studied dogs infected experimentally with both *Br. abortus* and *Br. suis*. Lesions which the authors felt were probably specific were observed in 1 dog inoculated intravenously with *Br. suis* and killed thirty-nine days later. Both kidneys of this animal were pale and areas of cortical scarring were present. The pelvis of each kidney contained a calculus. The prostate gland was hyperplastic and multiple purulent foci were present. However, changes in the liver and lung were the most suggestive of brucellosis. Foci of mononuclear or endothelial cells which had apparently arisen by proliferation of the cells lining the "intralobular capillaries" were seen in the liver. The larger vessels of the organ were not involved in this process. A solitary, tubercle-like lesion, in which acid-fast organisms were not demonstrable, was observed in the lung. The lesion was not encapsulated, but was well circumscribed by a peripheral zone of monocytes and lymphocytes. An epithelioid type of transition was observed in the central portion of the tubercle-like process. The authors concluded that the lung lesion was probably due to Brucella organisms.

Margolis and co-workers[3] reported upon the reaction of the reticuloendothelial system in experimental *Br. suis* infections in dogs. Severe clinical brucellosis was produced by repeated intravenous or intraperitoneal inoculations of 10 billion organisms. Granulomatous processes were observed in the lung, liver, spleen, kidneys, and lymph nodes. The most constant findings were seen in the lymph nodes and consisted of a pronounced reticuloendothelial reaction without significant enlargement of these organs. Epithelioid granulomata resulted from this process in time. Masses of large mononuclear, wandering cells eventually replaced the lymphatic channels. The observed nonspecific reactions, probably due to bacterial intoxication, included petechial hemorrhages, focal hyaline degeneration of the myocardium, and acute gastroenteritis.

Margolis *et al.*[4] observed a glomerulonephritis in dogs with experimental *Br. suis* infection. Focal epithelioid granulomata were found in the kidneys of 2 animals. In addition, an extensive fibrosis and hyalinization of the capillary loops, with resultant necrosis and proliferation of the capillary endothelium, were present. Capillary dilation, thrombosis, and glomerular hemorrhages were found in 2 other dogs. Adhesions of the glomerular loops involving the capsule were observed. Capsular thickening was present in some instances. Proliferation of the glomerular endothelium was reported, but this was not a prominent feature. A moderate degree of cloudy swelling was seen, but vascular changes were not observed.

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MATERIALS AND METHODS

Sections of lymph nodes from 6 Brucella-infected adult dogs of a previously reported investigation[5] (dogs 4, 5, 6, 8, 10, and 15) were prepared for histological examination. These nodes comprised pooled material from which *Br. abortus* had been isolated. Analogous tissues which did not contain Brucella were obtained from 5 apparently normal dogs and prepared for comparative studies.

In addition, tissues were obtained from young dogs which had the following histories. Seven animals were fed Brucella-infected milk and 3 of these (young dogs, 13, and 18)[6] were whelped by noninfected females; the others (young dogs 8, 9, 13, and 14)[6] were the offspring of Brucella-infected dams (adult dogs 6 and 8).[5] Four puppies were fed milk which did not contain Brucella or Brucella agglutinins. Two of these animals (young dogs 11 and 12)[6] were the young of infected dog 6 of a previous experiment;[6] while the other puppies were whelped by noninfected dams.

A consideration of the pathological alterations of the lymph nodes was the chief concern of this study, although specimens of the liver, kidney, spleen, and genital tracts were examined.

Tissues of the infected and control animals were fixed in a 10 per cent buffered formalin solution, pH 6.3 to 6.5, transferred through a series of butyl alcohol solutions, embedded in paraffin, and cut at 5 μ. Harris' hematoxylin and eosin stain or Mallory's triple stain was employed.

RESULTS

The types and relative proportions of microscopic abnormalities observed in the lymph nodes of Brucella-infected and normal dogs are shown in table 1, as are those of the puppies in table 2.

Granulomata were observed in 20 per cent of the lymph nodes of Brucella-infected animals, whereas none was encountered among the unexposed dogs. The lesions were composed chiefly of epithelioid cells surrounded by lymphocytes, eosinophils and/or reticular scarring (fig. 1). In some instances, there was a small area of central necrosis or central infiltration by polymorphonuclear or eosinophilic cells. Perhaps central necrosis had occurred regularly, but only occasionally were sections cut so that this region was included.

One granuloma was observed in a lymph node obtained from puppy 1 fed infected milk. None was found in the lymph nodes of puppies fed noninfected milk. The kidneys and liver of puppy 14, which was fed infected milk, contained several granulomatous lesions (fig. 2), while none was observed in the controls. This animal (young dog 14) was the offspring of an infected dam (dog 8).

Multinucleated cells were observed in approximately 15.5 per cent of the lymph nodes of adult dogs which had been fed infected material, as compared to approximately 2 per cent of the lymph nodes from control animals. The nuclei of these cells were centrally located. Binucleated or multinucleated cells were observed in 6 per cent of the lymph nodes from the puppies fed the infected milk. Cells of this type were not found in the tissues of the control puppies.

Reticular scarring was observed in 64.4 per cent of the lymph nodes of Brucella-infected dogs and in 74 per cent of the exposed puppies (fig. 3 and 4). The same phenomenon was seen in 76.5 per cent of

TABLE 1—Observed Pathological Alterations of Lymph Nodes of Adult Brucella-Infected* and Normal Dogs**

Abnormality observed	Brucella-infected dogs		Apparently normal unexposed dogs	
	Lymph nodes		Lymph nodes	
	(No.)	(%)	(No.)	(%)
Granulomata	9	20.0	0	0.0
Multinucleated cells	7	15.5	1	1.9
Reticular scarring	29	64.4	39	76.5
Hemosiderosis	44	97.8	19	37.3
Necrotic foci or abscess formation	27	60.0	39	76.5
Numerous plasma cells	32	71.1	18	35.3
Numerous polymorphonuclear cells	1	2.2	3	5.9
Numerous eosinophils	31	68.9	34	66.7
Fibrosis or fibrocytic proliferation	8	17.7	0	0.0
Phagocytosis of r.b.c.†	11	24.4	26	50.9

*Sections of 45 lymph nodes examined from 6 dogs. **Sections of 51 lymph nodes examined from 5 dogs. †Red blood cells.

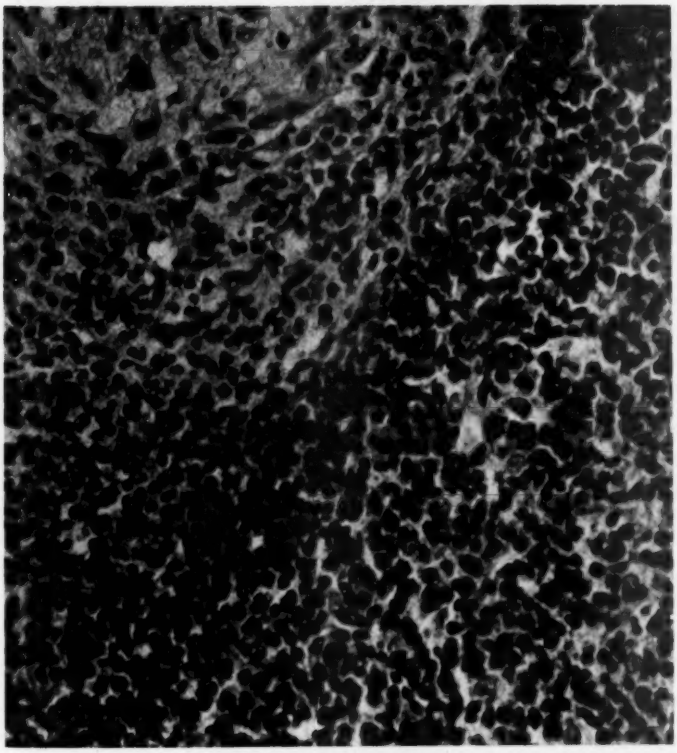

Fig. 1 — Granuloma surrounded by eosinophils of mesenteric lymph node of dog 10. x 600.

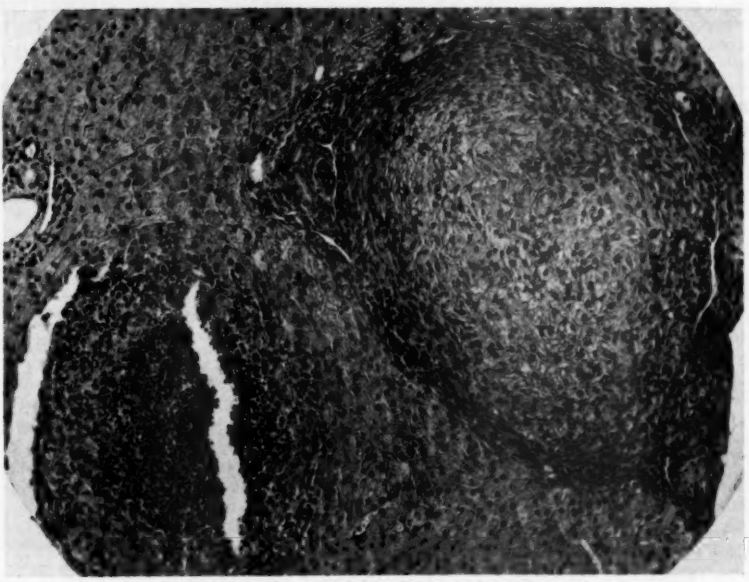

Fig. 2—Granuloma of liver of young dog 14. x 160.

the adult control dogs and in 76.9 per cent of the control puppies. Proliferative changes occurred in the same nodes in which atrophy and necrosis were present, thus suggesting that the process was continuous (fig. 4). Increased connective tissue without appreciable scarring was observed in some nodes.

The presence of hemosiderin was detected in 97.8 per cent of the lymph nodes of adult infected animals and in 37.3 per cent of the adult controls. It was present, also, in the lymph nodes of the exposed puppies (58%) and in their corresponding controls (23.1%).

Necrotic and abscessed areas were found in the lymph nodes of all groups of dogs examined. Necrobiosis was observed in 76.5 per cent of the lymph nodes of adult control animals and in 76.9 per cent of the nodes of the control puppies. This finding was observed in the infected adults' lymph nodes (60%) and in nodes of the puppies fed Brucella-infected milk (78%). Necrosis seemed to accompany or follow scarring before the advent of new tissue growth. Abscess formation and areas of polymorphonuclear infiltration were found in both the dogs and puppies given infected material. The bronchial-cervical group of lymph nodes of 1 of the infected adult dogs (dog 10) contained an area in which there was an extremely heavy concentration of lymphocytes surrounded by reticular scarring (fig. 3).

Plasma cells (fig. 5) were prominent in the lymph nodes of the infected adult dogs (71.1%) and in those of the puppies fed infected milk (66%). They were less prominent in the adult controls (35.3%) and in the control puppies (46.2%).

Eosinophils were observed in about equal numbers in the lymph nodes of the infected adult animals (68.9%) and in those of the adult controls (66.7%). They were present to a slightly greater extent in the control puppies (38.4%) than in the exposed puppies (34%).

Polymorphonuclear leukocytes were observed in the lymph nodes of 2.2 per cent of the infected adult animals and in 5.9 per cent of the control adults. They were seen in the lymph nodes of the puppies which were fed infected milk (4%) and in the corresponding controls (7.6%).

Phagocytosis of red blood cells (fig. 5) occurred more often in lymph nodes of the control adult dogs (50.9%) than in those of the Brucella-infected adult animals (24.4%). This process was observed in the lymph nodes of the puppies fed infected milk in 54 per cent of the nodes examined, and in those of the control puppies in 84.6 per cent of the nodes studied.

Table 2—Observed Pathological Alterations of Lymph Nodes of Puppies Fed Brucella-Infected Milk* and of Normal Puppies**

Abnormality observed	Exposed puppies		Control puppies	
	Lymph nodes (No.)	(%)	Lymph nodes (No.)	(%)
Granulomata	1	2.0	0	0.0
Multinucleated cells	3	6.0	0	0.0
Reticular scarring	37	74.0	10	76.9
Hemosiderosis	29	58.0	3	23.1
Necrotic foci or abscess formation	39	78.0	10	76.9
Numerous plasma cells	33	66.0	6	46.2
Numerous polymorphonuclear cells	2	4.0	1	7.6
Numerous eosinophils	17	34.0	5	38.4
Fibrosis or fibrocytic proliferation	0	0.0	0	0.0
Phagocytosis of r.b.c.†	27	54.0	11	84.6

*Sections of 50 lymph nodes from 7 puppies. **Sections of 13 lymph nodes from 3 puppies.
†Red blood cells.

DISCUSSION

Dogs travel freely from farm to farm among Brucella-infected cattle; they may drink infected milk and eat aborted material. One would, therefore, expect that dogs might serve as carriers of the disease and be of potential danger to noninfected animals. However, dogs apparently[2, 5, 6] have a high degree of natural immunity to *Br. abortus*. They may carry or drag infected materials from one pasture to another, thus endangering susceptible species. This probably represents the chief role of the

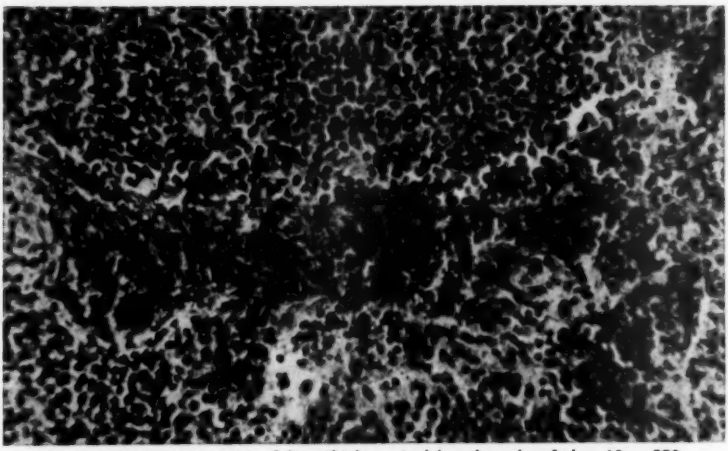

Fig. 3—Reticular scarring of bronchial cervical lymph node of dog 10. x 350.

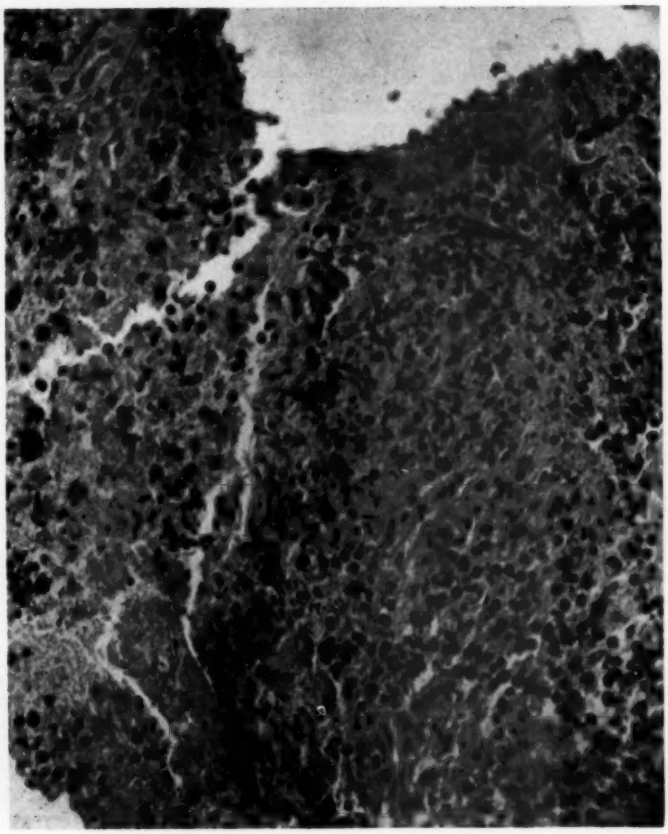

Fig. 4—Healing and scarring in bronchial lymph node of dog 4. x 400.

dog in the dissemination of brucellosis.

Certain pathological alterations observed in lymph nodes of animals which had been fed infected material for various periods were also found in the control dogs which were not exposed to Brucella. Apparently, lymph nodes of dogs undergo retrogressive changes which are not commonly found in other species of animals. Some of these alterations in other animals have been considered indicative of brucellosis. These changes in the dog can not be considered as a normal condition, neither can they be attributed to Brucella infection *per se*.

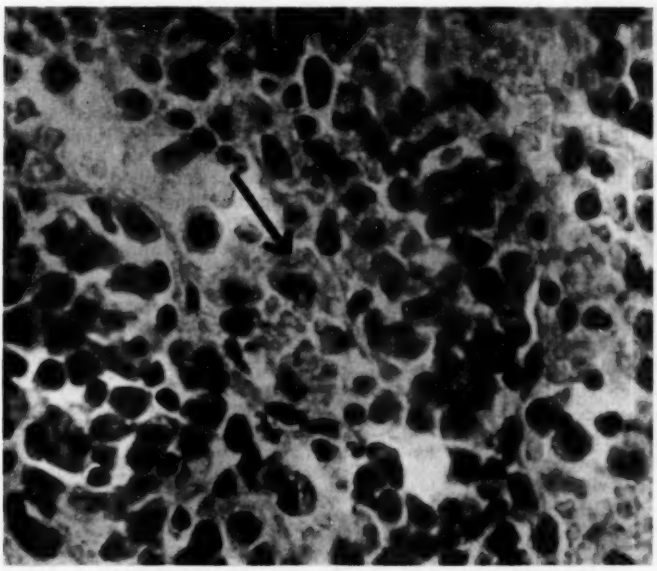

Fig. 5—Plasma cells and phagocytosis of red blood cells (arrow). Mesenteric lymph node of dog 10. x 450.

Granulomata and fibrocytic proliferation were not observed in the control animals. However, these two entities were encountered in the lymph nodes of the animals exposed to Brucella.

Granulomatous lesions are known to occur in many chronic disease processes. They are commonly observed in brucellosis of cattle, guinea pigs, and man. Their presence is not pathognomonic of the infection. Granulomata occurred in the lymph nodes, liver, and kidney of dogs fed material from Brucella-infected cows. These lesions were not found in the tissues of animals which were not exposed to *Br. abortus*. Their presence in the infected animals suggests that they were due to Brucella organisms. The lesions in these animals were apparently not progressive. This might be expected since the dog is not highly susceptible to the organism, the infections were localized, of long standing, and few, if any, Brucella were cultured from the tissues examined. The granulomata were small in size and involved all parts of the organ except the stroma.

Multinucleated cells of the lymph nodes respond to the presence of foreign materials and to some infectious processes. One might anticipate that these cells would react in an infection such as brucellosis. However, they were seldom observed and difficult to locate in the tissues of the infected dogs. An occasional cell was found in the material examined from the adult control animals. Hence, their significance in canine brucellosis is open to question.

Reticular scarring is often considered a characteristic feature of chronic inflammation. However, it may be a universal condition of the lymph nodes among dogs, since it occurred regularly in the control as well as in the infected or exposed animals. The lymph nodes of the puppies as well as the adult dogs showed the presence of reticular scarring, hence, age can not

be considered a determining factor in this case. Retrogressive and proliferative necrobiotic changes occurred in the same nodes without any marked change in the size of the node. The process in some instances appeared to be progressive. The presence of increased connective tissue without appreciable scarring might represent final healing or the end result of such changes. Perhaps reticular scarring has been too often attributed to Brucella infection.

The presence of hemosiderin in the tissues can not be related directly to the manifestation of Brucella infection, since the pigment has been found in the spleens of normal animals. Its presence is greatest in the horse and least in the dog, and usually greater in the old than in the young.

The presence of inflammatory cells, plasma cells, polymorphonuclear leukocytes, and eosinophils suggested that lymph nodes of dogs may be under some stress much of the time. Plasma cells seemed to be more abundant in normal dogs than had been previously reported. Polymorphonuclear leukocytes and eosinophils were found in the lesions of both infected and control animals; hence, their presence could not be attributed to Brucella infection.

The puppies were raised from the time of birth in cages which had wire netting as the floor. The adult dogs were housed in a similar manner throughout the course of these studies. Thus, it was assumed that the animals were not clinically parasitized and that the eosinophilic response was not attributable to the presence of intestinal parasites.

SUMMARY

A study was made of histological changes in the tissues of dogs fed materials of cows infected with *Brucella abortus*. Corresponding controls were also studied.

Most of the characteristic changes of the lymph nodes of dogs fed infected materials occurred in the lymph nodes of control dogs which were not exposed to *Br. abortus*. The two exceptions were the presence of granulomata (an involvement of 20%) and fibrocytic proliferation (17.7%).

Approximately 15 per cent of the lymph nodes of the adult dogs fed infected materials contained multinucleated cells, as compared to about 2 per cent of the controls. Cells of this nature were not observed in the tissues of control puppies, but binucleated or multinucleated cells appeared in about 6.0 per cent of the lymph nodes of the group fed infected milk.

Lymph nodes of both groups of dogs fed Brucella-infected materials, as well as the control animals, contained areas of reticular scarring, necrosis, abscess formation, hemosiderosis, plasma cells, polymorphonuclear leukocytes, and eosinophils.

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Some Aspects of Congenital Passive Immunity to Newcastle Disease in Chicks. I. The Transfer of Hemagglutinin Inhibitors from the Maternal Yolk to the Chick

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Literature

Congenital passive immunity is of decided epidemiological and immunological significance, and has been studied extensively in different animals. Among the more recent studies of this immunity in young mammals are those on African horse sickness,[9] *Salmonella abortus-equi*, hemolytic icterus,[7] and tetanus[14] in foals; on louping ill[20] and blue tongue[18] in lambs; on swine influenza[21, 22] in pigs; and on distemper[10] in puppies. In mammals, passive immunity can be transferred through the placenta or through the colostrum, or both, and Schneider and Szathmary[19] have correlated the method of transference with the structure of the placenta. There is no placental transfer in horses, cattle, swine, and goats, because these species have seven tissue layers between the maternal and fetal circulation. However, there is mainly placental transfer in rabbits, guinea pigs, mice, rats, and human beings, which have only three layers; and sheep and dogs take an intermediate position, with five and four layers of tissue, respectively, separating the two circulations.

In birds, the egg yolk appears to be the medium for the transference of passive immunity from the parent stock to the offspring. Studies of this process were conducted as early as 1893, according to Brandly and co-workers.[5] These authors have presented a full review of the literature on avian passive immunity up to recent years, and it appears superfluous to repeat it in this paper.

The above workers studied the congenital passive immunity of chicks to Newcastle disease (ND) and found that specific virus-neutralizing antibodies were present in the yolk of eggs laid by immune hens. Perceptible antibody activity by the serum of developing embryos could be first demonstrated in those 15 days old and increased activity at the eighteenth day of incubation. The immunity of chicks, the offspring of highly immune hens, was considerable for the first 29 days of their life, and some chicks remained refractory to infection up to 5 weeks. This study also demonstrated that congenital passive immunity interfered materially with an active immunity response of the chicks to vaccination or to infection. Intramuscular injections were used for challenge or vaccination experiments.

Chicks of immune dams from various sources were injected intramuscularly with virulent ND virus by Komarov.[12] Day-old chicks of different batches proved 47 to 100 per cent resistant; 1-week-old chicks, 50 to 60 per cent; and chicks 2 and 3 weeks old, 30 to 60 per cent. At 4 weeks of age, 25 to 27 per cent of them were still resistant, but none of 15, 6-week-old chicks survived. He also tested the interference of congenital passive immunity with the production of active immunity following vaccination with the "Mukteswar" live virus vaccine, and found that challenge at the age of 44 days killed 100 per cent of the chicks vaccinated when 1 day old, 40 per cent of those vaccinated at 1 week of age, but only 2 per cent of those vaccinated when 3 weeks old (vaccination and exposure by the intramuscular route).

Using chicks from breeding flocks vaccinated with different live virus ND vaccines or recovered from the disease proper, Alberts and Millen[1] demonstrated a high degree of immunity to a cutaneous-subcutaneous route (wing-web stick) of challenge exposure during the first 2 weeks of their life and a lower degree up to 4 weeks of age. The percentages of survivors from chicks of 20 flocks were: 91 per cent of 100, 1-day-old chicks; 87.4 per cent of 500, 7-day-old chicks; 77.2 per cent of 500, 14-day-old; 55.3 per cent of 513, 21-day-old; and 35 per cent of 282, 28-day-old chicks.

Jungherr and Terrel[11] demonstrated neutralizing antibodies in egg yolks from birds vaccinated against infectious bronchitis. In the developing embryos of such eggs, the yolk antibodies tended to decline while increasing in the tissues and the serum of the embryo. Chicks from vaccinated or naturally immunized parent stock maintained a high level of antibodies during the first two weeks of their life, but lost them by the fifth week.

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The purpose of this investigation was to study the passage of antibodies against ND from the dam to her chicks via the egg yolk, along lines similar to the work of Jungherr and Terrel with infectious bronchitis. However, since the serum-neutralization (SN) test is beyond the scope of our field laboratory, the hemagglutination-inhibition (HI) test was decided upon as a handy tool to trace this passage.

MATERIALS AND METHODS

Chickens and Eggs.—All eggs came from chickens kept either in individual laying cages (in the laboratory or on a breeding farm using the battery system plus artificial insemination) or in pedigree pens.* These hens had been vaccinated by the wing-web stick method, at 6 to 12 weeks of age, with the regular government-produced live virus vaccine used in this country,[12] but most hens chosen possessed an HI titer above the average.

For the sake of convenience and uniformity, we did not take into account the actual day and hour of hatch, but designated everything "embryo" until the completion of twenty-one days of incubation. Naturally, an embryo of 21 days was always a chick at least several hours old; and in this manner, every chick twenty-two days after the beginning of incubation was considered as a chick 1 day old.

Hemagglutination-Inhibition Test.—The procedure used was developed independently at this laboratory during the fall of 1947,[4] resembling the *beta* method of Brandly and coworkers[6] and the one described by Beach.[3] With this method, a titer of 1 : 20 is considered suspicious in diagnostic work but may represent complete resistance in a vaccinated bird. The comparability of results obtained at different dates was assured by the use of positive control serums (of known titers), which were common to the different tests.

Hemagglutination-Inhibition Test of Yolks.—The HI test was employed on yolks which had been previously extracted with ethylene dichloride as described by Schmittle and Millen.[16] Their original method plus refrigeration was completely satisfactory and more convenient for us than the later modification.[17] Due to the shortage of pure ethylene dichloride in this country, chloroform and carbontetrachloride were tried as fat solvents but without success; an aqueous fraction could not be separated from the emulsion. In embryos 20 to 21 days old and inside chicks, the yolk was semisolid and rather adherent to its surrounding yolk sac, rendering a clean separation of the two nearly impossible. Therefore, both were ground together in a mortar, after the addition of measured amounts of saline, and the supernatant fluid was used. The dilution was based on the original volume of both components without compensating for the inert membranes. This may be the reason for relatively low values in chicks 3 to 5 days old, when the yolk sac membranes may constitute half of the total volume. No interference was noticed by normal hemagglutinins possibly present in these membranes,* and with the previously mentioned methods, neither hemagglutinins nor hemagglutinin-inhibiting factors could be demonstrated in yolk sac membranes of embryos 19 to 21 days old.

Hemagglutination-Inhibition Tests of Livers.—Embryo and chick livers, or parts of them, were measured in a 10-cc. graduate cylinder by the addition of a known quantity of physiological saline solution, ground in a mortar, and then diluted 1 : 10. With embryos up to 18 days, the supernatant fluid was used directly for the HI test, whereas livers of later stages were extracted with ethylene dichloride after the grinding and before centrifugation, in a manner corresponding to that for yolk extraction.

RESULTS AND DISCUSSION

Comparison of Hemagglutination-Inhibition Titers of Blood Serums and of Egg Yolks.—At the onset of this study, it seemed an essential requisite to make a preliminary comparison between the serum titers of hens and the yolk titers of their eggs over an extended period, before comparing eggs or embryos of the same hen produced at different dates.

Comparisons between serum and yolk titers have been reported by Schmittle and Millen,[18] Schmittle,[17] and Doll *et al.*[8] The two former studies include only single comparisons per bird and show a very high correlation. In the latter work, a comparison of several eggs per hen was made, unfortunately, however, during a period of rapid fall of the HI titers in the serum of the hens. Therefore, these data are not fully conclusive.

Table 1 presents some typical comparisons, including 1 hen with a very high titer and 1 with an insignificant one. Although the number of hens involved is small, it is apparent that within each period tested the differences between the serum titer and those of the corresponding yolks, or among the yolks themselves, did not exceed one dilution—that is, they ranged

*The authors take this opportunity to thank Mr. Einhorn of Holon and the poultrymen of Kvuzat Yavneh for their coöperation and generosity of donating most of the embryonated eggs used in this study.

within the limits of experimental error. Moreover, in case of differences between serums and their corresponding yolks, the yolks always possessed the higher titer. This tendency is also apparent in table 2, and is not in agreement with the results of Schmittle.[17] He found that serum and yolk titers were the same in more than 55 per cent of the cases, that the serum titer was twice the yolk titer in 22 to 43 per cent, and that the yolk titer was only rarely twice the serum titer. The reason for our relatively high yolk titers might be found in the accidental inclusion of some albumin with the yolk samples, before realizing that the former has an agglutination-inhibitory effect, as demonstrated by Schmittle.[17]

Hemagglutination-Inhibition Tests of Liver Extracts and Allantoamniotic Fluids.—It had been hoped that embryonic liver tissue might contain hemagglutinin-inhibiting antibodies, and that it could be used to trace the latter in young embryos not containing enough blood serum for testing purposes. However, as a rule, no HI titers were obtained with the technique employed. In 1 or 2 exceptional cases, the titer found was assumed to be due to "contamination" with an antibody-containing substance.

Equally negative results were obtained with allantoamniotic fluids. These negative results agree with those of Brandly and co-workers,[5] who found only negligible amounts of virus-neutralizing antibodies in allantoic and amniotic fluids.

The Transfer of Hemagglutination-Inhibition Antibodies from the Maternal Yolk to the Serum of Embryos and Chicks.—The yolks and blood serums of embryos and chicks of 12 hens were tested systematically by means of the HI test, in order to trace the transfer of antibodies from the yolk of the dam to the blood of her chick. The results are summarized in tables 2, 3, 4, 5 and in fig. 1. Although there were not available enough embryos and chicks to test all desirable stages of the offspring of every hen, a number of points emerge rather clearly.

The increase in the serum titers of embryos and of chicks does not take place at the expense of maternal yolk titers, which remain rather constant throughout the incubation period and through the first five days of the chicks' lives. In our opinion, the decrease in yolk titers appearing in a few cases after hatching is due to the technical difficulties explained under the description of HI tests of yolks. As a rule, after 4 days of age, the unabsorbed yolk became too small and too solidified for an HI test.

These unchanging yolk titers, in spite of

TABLE I—Comparison Between Hemagglutination-Inhibition Titers of Serums and Egg Yolks of Newcastle Disease Immune Hens

Hen (No.)		8476			6039			313			834		
Date of test	Date of material	Serum titer	Yolk titer	Date of material	Serum titer	Yolk titer	Date of material	Serum titer	Yolk titer	Date of material	Serum titer	Yolk titer
10/31/49	10/4	.....	10,240	....	..	...	....	....	....	....	..	...
	10/30	10,240	20,480	....	..	...	....	....	....	....	..	...
	11/4	.....	>20,480	11/2	..	160	11/4	....	1,280	11/1	..	<40
	11/6	.....	>20,480	11/4	..	160	11/7	....	>1,280	11/3	..	<40
11/9/49	11/8	20,480	.....	11/6	..	80	11/8	1,280	....	11/6	..	<40
				11/8	80	80	....	....	....	11/7	..	<40
										11/8	<10	...
11/17/49	....	.....	.....	11/12	..	80	11/13	....	2,560	....	..	...
	....	.....	.....	11/13	..	160	....	....	....	....	..	...
	....	.....	.....	....	..	...	11/10	....	2,560	11/17	..	<10
	....	.....	.....	....	..	...	11/17	....	2,560	11/20	..	< 5
11/23/49	....	.....	.....	....	..	...	11/21	....	1,280	11/22	< 5	...
	....	.....	.....	....	..	...	11/22	1,280	....	....	..	...
	12/4*	.....	>20,480	12/6*	..	80	12/5*	....	5,120	11/25	..	10
	12/5	.....	>20,480	12/8	..	80	12/9	....	5,120	12/3*	..	2.5
12/15/49	12/7	.....	>20,480	12/14	80	...	12/13	....	2,560	12/6	..	5
	12/14	>20,480	.....				12/14	2,560	....	12/13	..	2.5
										12/14	2.5	...

*Fresh, infertile egg placed into incubator for seven days and held one additional day at room temperature.

a simultaneous increase in serum titers, differed from the results obtained by Jungherr and Terrel[11] in their investigation of passive immunity to infectious bronchitis by means of the SN test.

TABLE 2—Yolk Titers of Embryos at Various Stages of Development

Dam (No.)	4011	1	1497	313	6	4
Serum titer of dam	320	640	640	1,280	2,560	..
Titer of infertile egg	320	...	1,280	....	2,560	..
Age of embryo (days)						
10	...	...	....	1,280	....	..
11	...	...	....	1,280	....	..
12	...	...	1,280	1,280	....	..
13	...	...	....	1,280	....	..
14	320	...	....	1,280	....	..
15	...	...	....	640	....	..
16	320	320	....	1,280	1,280	80
17	...	...	....	....	....	80
18	320	...	1,280	....	....	80
19	320	320	....	....	....	80
20	640	320	1,280	....	1,280	80
21	320	640	1,280	....	....	40

Significant serum HI titers appeared rather late during embryonic development and only close to hatching time, except in cases of very high maternal titers. Brandly and co-workers[5] were able to demonstrate some virus neutralization as early as the fifteenth day of incubation and significant neutralizing titers after eighteen days. This may be due to the possibility that SN antibodies are demonstrable earlier than HI antibodies, as shown by Doll *et al.*[8] for experimentally exposed chickens, or else they may have used dams with higher antibody content than the routinely vaccinated birds employed by us. Great difficulties were encountered in collecting sufficient blood for HI tests from individual embroyos less than 20 days old, and there were not available enough embryonated eggs for pooled samples, hence, the first appearance of HI antibodies in embryonic serum could not be established by the methods employed.

An interesting point related to the previously mentioned late development of significant titers is the fact that, in the majority of chicks tested, the titers reached their highest value only at the age of 2 days or older, and not at hatching time (embryos 20 to 21 days old) when the titer may be only one fourth of it or less. Shortly after the peak titer had been reached, there began a relatively fast decline. After two weeks, there were hardly any significant titers left, even in the offspring of dams with rather high titers. Levine and Fabricant[15] found practically no significant HI and SN titers in chicks 17 days old.

The above workers[15] also compared the SN titers of 20 dams with 1 of each of their day-old chicks and found them practically identical. Our data on HI titers do not conform with these results. As pointed out in the previous paragraph and in table 5,

TABLE 3—Blood and Yolk Titers of Embryos and Chicks

Dam (No.)		1497		1		4011		1046		1037		1044	
Material tested		Blood	Yolk	Blood	Yolk	Blood	Yolk	Blood	Yolk	Blood	Yolk	Blood	Yolk
Titer of dam		640	....	640	...	320	...	...	640	...	320	...	320
Age (days) of													
Embryo	Chick												
18		40	1,280	...	...	20	320	...	...	...	...	...	...
19		80	....	...	320	10	320	...	...	...	...	...	...
20		80	1,280	20	320	20	640	20	160	...	...	20	80
21		80	1,280	20	640	40	320	40	320	40	160	20	...
	1	320	1,280	80	320	80	320	80	320	40	160	40	80
	2	320	640	160	640	160	320	80	160	80	80	80	80
	3	320	320	...	...	160	160	160	†	80	†	160	†
	4	640	....	160	320	160	160	160	...	40	...	80	80
	5	320	....	...	...*	80	...	160	...	40	...	80	...
	6	...	....	80	...	...	...	...	...	...	...	...	...
	7	320*	....	...	...	80*	...	80*	...	40*	...	80*	...
	8	...	....	40	...	...	...	...	...	...	...	...	...
	10	160	....	...	...	40	...	40**	...	40	...	20	...
	14	80*	....	...	...	20*	...	...	...	10*	...	10*	...
	19	20**	....	...	...	10	...	...	...	...	...	5	...

*Second bleeding of same chick. **Third bleeding of same chick. †Yolk extraction unsatisfactory.

day-old chicks had not reached yet their highest values. Moreover, even at their top values one to three days later, only about half of the chicks reached the titer of their dams. Figure 1 presents a typical distribu-

trends within the table, tend to emphasize the reliability of the results obtained.

We were able to demonstrate repeatedly this reliability, that is, the fact that the offspring of a given dam had a certain rela-

TABLE 4—Blood and Yolk Titers of Young Chicks

Dam (No.)		302		1010		1164		1060		3		301	
Material tested		Blood	Yolk	Blood	Yolk	Blood	Yolk	Blood	Yolk	Blood	Yolk	Blood	Yolk
Titer of dam		...	....	...	160	...	160	...	160	80	...	...	...
Age (days) of													
Embryo	Chick												
20		...	160	10	80	5	...	...	...	<20	80	...	40
21		40	160	10	...	10	160	20	...	10	80	10	80
	1	160	160	80	160	20	80	10	80	10	160	20	80
	2	...	....	80	160	20	80	10	80	20	160	20	40
	3	160	160	160	160	10	80	20	†	40	160	40	40
	4	...	....	80	...	80	80	40	80	...	...	...	...
	5	160	....	80	...	40	...	40	...	...	...	40	...
	7	80	....	40*	...	20	...	20*	...	20	...	20*	...
	10	40*	....	20	...	...	...	10	...	...	...	20**	...
	14	20**	....	10*	...	...	...	5*	...	...	...	...	...
	19	20	....	5**	...	...	...	5**	...	...	...	...	...

*Second bleeding of same chick. **Third bleeding of same chick. †Yolk extraction unsatisfactory.

tion of serum and yolk titers among the offspring of 1 hen.

In table 5, the serum titers of chicks of various ages are expressed as a fraction of the highest titer found among the offspring of a certain dam, rather than as their numerical values. Among the chicks of dam 1497, the highest titer was 640; hence, a titer of 40 is expressed as 1/16 maximal titer, or a titer of 80 as 1/8 maximal titer. In this manner, the rise and fall of titers at the various stages of development are correlated independently of the quantitative differences between different chicks at the same age. This manner of comparison might be applicable to other vaccines and to other techniques of the HI test. The logical distribution of the titers within one age group, and of the various age group tively invariable congenital titer at each stage of their development as long as the maternal titer remain unchanged. For instance, chicks of dam 1010 were blood-tested when 3 days old on 1/14/51, 3/18/51, and 4/15/51 (hatches of 1/10, 3/14, and 4/9, respectively), and the serum titers obtained were 80, 160, and 160, respectively. On 3/18/51, chicks of dams 1037 and 1046 were tested at an age of 2 and 4 days, and offspring of a later hatch supplied the 3- and 5-day-old stages which were tested on 4/15/51, still they supplemented each other accurately. The hatch of 1/10/51 supplied enough chicks of hens 1044 and 1060 for a series of tests up to 7 days of age, except for the 4-day-old stage. This missing age was added from the hatch of 2/1/51 and tested at a correspondingly later date,

TABLE 5—Chick Serum Titers at Various Ages Expressed as Fraction of Maximal Titers*

Age (days) of		No. serums fitting each fraction						Total No. serums of that age	Average** fraction at that age
Embryo	Chick	1/32	1/16	1/8	1/4	1/2	1/1		
20			2	5	1	..	..	8	1/8
21			1	4	5	2	..	12	1/4
	1	..	..	..	4	7	1	12	1/2
	2	..	..	..	2	6	3	9	1/2
	3	..	..	1	..	2	8	11	1/1
	4	..	..	..	..	3	6	9	1/1
	5	..	..	..	..	6	4	10	1/2
	7	..	..	..	2	8	..	10	1/2
	10	..	..	2	5	2	..	9	1/4
	14	..	2	5	..	..	..	7	1/8
	19	3	2	1	..	..	..	6	1/32

*Based on tables 3 and 4. **Mode, that is, the most frequent value.

nevertheless the latter values fit in perfectly with the former ones. As a further test, yolk and blood samples of 4, 1-day-old chicks of 4 dams were tested during June, 1951, and the results compared with those ob-

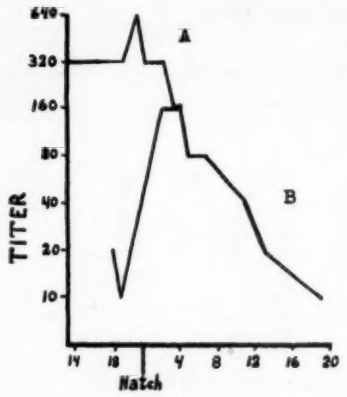

Fig. 1—Hemagglutination-inhibition titers of chicks hatched from hen 4011, whose serum titer was 320. A = yolk titers; B = serum titers.

tained in March. The corresponding titers were either identical or the differences between them did not exceed one dilution; that is, they ranged within the limits of experimental error.

SUMMARY AND CONCLUSIONS

1) The hemagglutination-inhibition (HI) titers of egg yolks are a reliable quantitative index of the serum titers of the hens laying them. Both titers are almost identical, within range of experimental error.

2) Hemagglutinin - inhibiting antibodies were not found in saline-liver suspensions nor in allantoamniotic fluids of developing embryos whose yolks contained significant amounts of them.

3) The maternal yolk titer remains constant during incubation, and the serum titer of the embryo does not ascend at its expense.

4) Significant HI embryonic serum titers appeared only near hatching time, except when the maternal titers were very high.

5) At hatching time, serum titers of chicks had reached only one eighth to one fourth of their highest value, which generally is reached at 2 to 3 days of age. This peak value does not necessarily equal the maternal titer and may be significantly lower.

6) Chicks of a given dam have a quantitatively specific titer for each stage of their development, and this titer seems to be constant as long as the maternal titer does not change.

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Some Aspects of Congenital Passive Immunity to Newcastle Disease in Chicks. II. The Relationship of Maternal Hemagglutination-Inhibition Titers in Baby Chicks to their Actual Immunity

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THE TRANSFER of hemagglutinin-inhibiting antibodies from the maternal yolk to the chick through the developing embryo has been described in a preceding paper,[3] but the interpretation of these titers in terms of actual immunity has become a controversial subject. Since the above investigation was started, a number of studies have been published which raise serious doubts as to the significance of passive immunity in chicks, both as a protection against virulent infection and as a condition interfering with the development of active immunity.

LITERATURE

The above publications deal with passive immunity to both Newcastle disease (ND) as well as to infectious bronchitis. In the latter case, Hofstad and Kenzy[9] have shown that, although a high serum antibody level existed in chicks hatched from hens recovered from bronchitis, this did not prevent typical active infection during their first ten days of life. The authors conclude that "it is possible that complete immunity to bronchitis may depend on interaction between tissue immunity and circulating antibodies."

With regard to ND, Olson and co-workers[11] observed five outbreaks of this disease in chicks less than 1 week old and within seven days of arrival on the farm, although they came from a hatchery the supply flocks of which had been vaccinated effectively with commercial live virus vaccines. Hitchner[6] used chicks hatched from hens which had been vaccinated 126 days earlier with a commercial live virus vaccine. Although a pooled serum sample of 11 chicks revealed a hemagglutination-inhibition (HI) titer of 160, 76 per cent of 25 chicks challenged intranasally became infected, and 54 chicks vaccinated intranasally when 1 day old proved solidly immune at 5 weeks of age, as compared to 70 per cent infection among the controls. Doll *et al.*[4] demonstrated that in chicks of vaccinated hens, or even of hens which had survived a natural outbreak, "an immunizing reaction occurred in parentally immune chicks at all ages from 1 day upward following intranasal instillation of living Newcastle disease virus." They believe that passive immunity does not prevent infection but causes a variable modification of the infection.

Hitchner[6] and Doll *et al.*[5, 6] showed that chicks from dams which had been vaccinated with the Hitchner (B1) strain were not refractory to virulent or modified virus of the vaccine. Some of this susceptibility may be due to the absence of congenital antibodies in such offsprings,[6] but Hitchner found that these chicks were not immune to intransal exposure of either one of these viruses in spite of significant passively conferred HI titers. Markham, *et al.*[10] reported that "one- or two-day-old chicks that have demonstrable antibody in their circulation can be successfully vaccinated, and such birds are shown to withstand intranasal challenge with highly virulent field virus at five, seven and ten weeks of age." Vaccination was performed either intranasally or by the conjunctival route with the Blacksburg (Hitchner) strain.

Employing both HI and serum-neutralization tests in hens and chicks and working under carefully controlled conditions, Levine and Fabricant[12] came to the conclusion that "all the chicks, whether they had high titers or not, succumbed to respiratory infection when challenged with ND virus dropped in the nares." They point out a similar situation with human influenza, which may attack people in spite of their high antibody titer. The same seems to hold true for swine influenza, too. Young[15] states that "circulating antibody alone has been shown incapable of warding off infection; it only modifies the disease by decreasing its intensity."

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A comparison of the above studies, which resulted in negative opinions about the practical significance of passive immunity[5, 6, 8, 12, 13, 14] with the positive results demonstrated by Brandly *et al.*,[4] Komarov,[10] and Alberts and Millen,[1] who found a distinct correlation between passive immunity and actual resistance, brings out one difference which is common to both groups and which might be decisive: The former negative results were obtained following exposure by the *nasal route* (assuming that natural infection, too, is primarily a respiratory one), while the latter positive results were demonstrated after *intramuscular* injection or *cutaneous* stick. In view of the possibility that the contradictory results might be due to the difference in the route of vaccination or exposure, it was decided to compare the nasal and intramuscular routes of vaccine application in chicks of known congenital HI titers.

MATERIALS AND METHODS

Challenge Virus.—This virus (isolated from a natural outbreak in the field) was obtained in the form of allantoamniotic (a.a.) fluid from Dr. A. Komarov, Virus Disease Section, Israel Veterinary Institute. One and the same test tube of this fluid was used throughout the various trials, and a preliminary test showed that 0.1 cc. of a 1 : 1,000 dilution (lowest dilution tried) instilled intranasally killed 4½-week-old cockerels within seven days.

Vaccine.—Two ampules were picked at random from the stock of regular government-produced live virus vaccine[11] held for general distribution and mixed together.

Vaccination Experiment.—Twenty-five day-old chicks, the offspring of 8 dams, were vaccinated with ¼-adult dose, 13 of them intramuscularly and 12 by intranasal instillation. Both groups were housed in separate cages after addition of 5, 1-day-old unvaccinated controls. The cages were placed one on top of the other to equalize environmental conditions to some extent. The HI titers of these chicks had been established in previous experiments.[3] They were bled when 25 days old, and two days later were challenged intranasally with 0.1 cc. of 1 : 10 virulent a.a. fluid. Two 6-week-old cockerels served as virus controls, 1 receiving the above dose and the other one tenth of it.

RESULTS AND DISCUSSION

This experiment (summarized in table 1) was designed to compare the efficiency of intramuscular and intranasal vaccination of chicks with parentally conferred serum HI titers. According to this setup, four criteria are available for measuring the success or failure of the vaccination. For in-

TABLE 1—Results of Vaccinating 1-Day-Old Chicks and Thereafter Challenging Them Intranasally at 27 Days of Age

Dam (No.)	1046	1037	1010	1054	1175	1052	1163	1178	1058	4011
HI titer of:										
dam's yolk	640	320	160	160	160	80	40	40	80	320
day-old chick	80	40	80	40	40	20	5	5	20	80
Intramuscular vaccination										
No. of chicks	2	2	2	2	2	1	1	1	....	....
HI of chicks 24 days after vaccination	<20	....	2 x <20	<20	<20	<20	....	....	....	....
Mortality:										
nonspecific	1	2	1	1	2	....	1	1	....	....
Newcastle disease	1	....	....	....	....	....	....	....	....	....
HI of survivors 12 days after challenge	....	....	>2,560	2,560*	....	>2,560	....	....	....	....
Intranasal vaccination										
No. of chicks	1	2	2	2	2	1	1	1	....	....
HI of chicks 24 days after vaccination	<20	640	160	2 x 160	....	320	....	320	....	....
Mortality:										
nonspecific	1	1	1	1	2	....	1	1	....	....
Newcastle disease	...	....	....	....	....	....	....	....	....	....
HI of survivors 12 days after challenge	...	320	160	1,280	....	640	....	....	....	....
Nonvaccinated controls, 1 chick each										
HI at 25 days of age	...	....	....	....	<20	....	....	....	....	<20
Mortality:										
nonspecific	...	....	....	1†	....	1†	....	....	1†	....
Newcastle disease	...	....	....	....	1‡	....	....	....	....	1‡

*Survived after showing symptoms. †Controls housed together with those vaccinated intranasally. ‡Controls housed together with those vaccinated intramuscularly.

stance, should vaccination be successful in spite of passive immunity, we should expect the following results: (1) a postvaccination reaction five to ten days after vaccination; (2) a high postvaccination HI titer twenty-four days after vaccination; (3) no morbidity or mortality four to ten days after challenge; and, (4) no significant increase in the HI titers twelve days postchallenge as compared to the postvaccination titer of fourteen days earlier.

On the other hand, should passive immunity interfere with active immunization, there should be neither a postvaccination reaction nor a high postvaccination titer. However, we should expect some postchallenge morbidity or mortality and a significant increase in the HI titers of the survivors of the challenge.

In the group vaccinated *intramuscularly*, 1 chick died during the first four days after vaccination, 1 chick died during the period of potential postvaccination reaction, and symptoms were not seen in the other chicks. Five chicks died during the period ten to twenty-five days after hatching. None of the 6 chicks blood-tested showed a significant HI titer twenty-four days after vaccination. Two of these died before and immediately after the intranasal challenge exposure; therefore, only 4 reached the postchallenge period. Of these, 1 died, 1 showed typical respiratory symptoms but survived, and 2 did not appear to react at all. However, all 3 survivors possessed very high HI titers twelve days after the challenge, proving that the latter 2 chicks also had passed through an active infection. The 2 unvaccinated controls housed in this cage did not show significant titers at 25 days of age, and both succumbed to the exposure. These results prove that intramuscular vaccination of passively immune chicks was ineffective in this experiment.

In the group vaccinated intranasally, 1 chick died during the first four days after vaccination. During the period of potential postvaccination reaction, neither respiratory nor nervous symptoms were observed, but 4 vaccinated chicks and 1 unvaccinated control died, and during the period of ten to twenty-five days of age, there was no mortality among the vaccinated chicks but 2 more control chicks died. Twenty-four days after vaccination, 1 chick lacked significant HI titer; however, the 6 others had developed high titers (titers above 20 are significant). The chick with the negative titer died one day before the intranasal exposure, but none of the 6 challenged chicks died or developed any symptoms. Unfortunately, 2 of these survivors could not be blood-tested, because they died of coccidiosis ten and eleven days after exposure, that is, at least four days after the deaths caused by ND among the chicks of the intramuscular group or among the 6-week-old controls. Only 1 out of the 4 chicks blood-tested twelve days after the challenge yielded an HI titer which had been increased significantly due to this exposure.

Comparing the results of intranasal vaccination on the basis of the four criteria established previously, the conclusion seems justified that, in the majority of cases, this method of immunization was effective in spite of significant congenital HI titers at the time of vaccination. Since the number of chicks involved was small and since the high nonspecific mortality interfered seriously with evaluation of the results obtained with this group of chicks, these results can not be presented as fully conclusive; however, they are in full agreement with the literature cited.

The results of this vaccination experiment indicate that passive immunity neutralizes efficiently a live virus vaccine applied intramuscularly, and that this route should not be used, therefore, in young chicks which are the offspring of dams possessing a decided immunity as evidenced by a significant level of circulating antibody. This is in full agreement with the results obtained by Brandly and coworkers[4] and Komarov.[10] However, it might be that not all vaccinated chickens necessarily retain such a high degree of immunity until they are used for breeding purposes.[2, 5, 6, 7, 13] This experiment also indicates that passive immunity does not necessarily interfere appreciably with a vaccine applied by nasal instillation, and that this route should, therefore, be the preferred one for vaccinating baby chicks suspected of possessing congenital passive immunity. These results agree with those of Hitchner,[8] Doll *et al.*,[6] and Markham *et al.*[13]

It is suggested that the above experiment be repeated on a larger scale and under more favorable conditions, in order to compare and clarify more definitely the influence of the two routes of application of vaccines upon the efficiency of congenital passive immunity.

SUMMARY AND CONCLUSION

Chicks with congenital passive immunity appear refractory to intramuscular vaccination; however, the same chicks vaccinated with an identical dose by nasal instillation seem to develop infection and a subsequent satisfactory immunity, as indicated by high hemagglutination-inhibition titers and resistance to intranasal challenge exposure.

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Chemotherapy of Enterohepatitis of Turkeys. I. The Value of 2-Amino-5-Nitrothiazole in Prevention and Treatment

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RECENTLY, THERE HAS been a revived interest in the study of enterohepatitis (blackhead) in turkeys. Much of this interest has been stimulated by the reports of effective chemotherapeutic agents. DeVolt and Holst[1, 2] reported that 5-chloro, 7-iodo, 8-hydroxyquinoline (vioform, Ciba) and 5-chloro, 8-hydroxyquinoline (clioform, Ciba) were effective in reducing blackhead mortality. Sautter *et al.*[3] found that stovarsol, tryparsamide, and vioform had protective value. Waletzky *et al.*[4] reported that enheptin-T and enheptin-P (American Cyanamide Co.) practically prevented mortality in young poults inoculated with infected liver material. Jungherr and Winn[5] reported field studies with enheptin-T. These workers stated that 0.05 per cent of the drug in the ration was effective as a preventive and that 0.1 per cent was effective as a treatment for enterohepatitis.

The purpose of this paper is to report an evaluation of 2-amino-5-nitrothiazole in the treatment and prevention of enterohepatitis in turkeys under simulated field conditions.

MATERIALS AND METHODS

The birds used in this study were 7-week-old, Broad Breasted Bronze poults that were obtained from a commercial turkey producer. The holding facilities consisted of 20, 16-by-16-ft. tents. Adjoining each tent was a 16-by-20-ft. pen. All tents were erected on ground that had been used for several years as a range for chickens.

Fifty poults of both sexes, selected by random sampling, were placed in each tent. Since the birds were 7 weeks old at the beginning of the experiment, heating facilities were not needed. Throughout the experiment, the birds were given an appropriate all-mash turkey feed. The drug was mixed with the feed on the farm by using a ½-ton electrical feed mixer.

The experiment was comprised of three parts. Part 1 was designed to determine the prophylactic value of 2-amino-5-nitrothiazole when medication was started at the time of exposure to infection and given continuously in the feed. It involved six pens identified as A, B, C, D, E, and F. Pens A through E received the drug at the following concentrations: 0.05, 0.04, 0.03, 0.02, and 0.01 per cent of the ration, respectively; pen F served as the nonmedicated infected control.

Parts 2 and 3 were designed to ascertain the value of the compound in controlling enterohepatitis when medication was started after an outbreak of the disease had occurred. Part 2 was comprised of pens G, H, J, K, L, and M. Pens G through L received drug concentrations of: 0.05, 0.04, 0.03, 0.02, and 0.01 per cent of the ration, respectively. Medication was continuous for twenty-one days. Pen M served as the nonmedicated infected control.

In Part 3, medication was delayed until enterohepatitis had appeared in the pen. Pens N, P, R, S, T, and U received medication, while pen X served as the nonmedicated infected control group. In this test, however, medication was given for varying periods of time; in some pens according to an intermittent schedule of treatment. The amount and schedules of medication were: pen N, 0.1 per cent for fourteen days; pen P, 0.1 per cent for seven days; pen R, 0.1 per cent according to a three-three-three* intermittent schedule; pen S, 0.05 per cent for fourteen days; pen T, 0.05 per cent for seven days; and pen U, 0.05 per cent according to a three-three-three intermittent schedule.

The original intention was to allow the ground which had been used as a chicken range to serve as the source of infection in parts 1 and 3 of this study. In addition to being on the contaminated ground, the birds in part 2 were given approximately 50 embryonated *Heterakis gallinae* eggs per bird as an added source of histomonad exposure when they were

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We are indebted to Merck and Company, Inc., Rahway, N. J., for the drugs and financial aid for this study.

*The birds were given medicated feed for three days, nonmedicated for three days, and again given medicated feed for three days.

10 weeks old. The eggs were administered by means of a glass syringe to which a piece of rubber tubing was attached.

Neither the ground that had been used as a range for chickens nor the *H. gallinae* eggs produced the quantity or uniformity of histomonad infection desired, therefore chicken litter from a chicken hen house was placed in each pen when the poults were 14 weeks old.

The birds were identified by wing bands at the beginning of the experiment. Individual bird weights and feed and water consumption for each pen were obtained weekly. Each bird that died was examined postmortem and an attempt made to determine the cause of death. The diagnosis of enterohepatitis, when made, was based on gross lesions. The study was concluded when the birds were 22 weeks old.

DISCUSSION

Ground that had been recently used as a range for chickens did not prove a reliable source of enterohepatitis infection; nor did the 50 embryonated *H. gallinae* eggs per bird in part 2 provide sufficient histomonad infection for evaluating the drug treatment. The chicken litter apparently harbored sufficient *Histomonas meleagridis* to produce a uniform infection; however, the variable infection and scattered mortality in the early stages of parts 2 and 3 of the experiment make it difficult to interpret the results of the drug treatment.

The mortality in part 2, where the birds received 50 embryonated *H. gallinae* eggs, was consistently lower even after the chicken litter was introduced. This suggests the possibility of some infection from the histomonad-carrying *H. gallinae* eggs and the subsequent development of some resistance to enterohepatitis.

RESULTS

Table 1 summarizes the data on medication, per cent of enterohepatitis mortality, average weight per bird, and the pounds

TABLE I—The Use of 2-Amino-5-Nitrothiazole in the Prevention and Treatment of Enterohepatitis (Blackhead) in Turkeys

Part 1

Medication started at time of exposure and given continuously for thirteen weeks

Pen	A	B	C	D	E	F
Medication (concentration)	0.05	0.04	0.03	0.02	0.01	None (control)
Blackhead mortality (%)	0	0	2	8	20	44
Ave. wt. per bird	12.20	12.70	12.30	12.70	12.60	13.20
Lb. feed per lb. gain	6.93	5.54	5.20	4.90	4.96	5.25

Part 2

Medication started after outbreak and given continuously for twenty-one days

Pen	G	H	J	K	L	M
Medication (concentration)	0.05	0.04	0.03	0.02	0.01	None (control)
Blackhead mortality (%)	10	2	32	20	12	6
Ave. wt. per bird	13.20	13.05	13.50	13.05	12.90	13.30
Lb. feed per lb. gain	5.05	5.15	5.15	5.15	5.50	5.26

Part 3

Medication started after outbreak

Pen	N	P	R	S	T	U	X
Medication (concentration)	0.1 for 14 days	0.1 for 7 days	0.1 according to a 3-3-3 intermittent schedule	0.05 for 14 days	0.05 for 7 days	0.05 according to a 3-3-3 intermittent schedule	None (control)
Blackhead mortality (%)	38	22	34	36	32	30	24
Ave. wt. per bird	12.50	12.50	11.90	12.60	11.90	12.05	11.90
Lb. feed per lb. gain	5.04	6.42	5.70	5.35	5.53	6.26	6.35

of feed required to produce a pound of gain.

It is apparent from these data that 2-amino-5-nitrothiazole given continuously in an all-mash ration at a concentration of 0.05, 0.04, and 0.03 per cent as a prophylactic measure was effective in reducing mortality from enterohepatitis. There was no mortality from enterohepatitis in the birds receiving 0.05 and 0.04 per cent of the drug and only a 2 per cent mortality in the birds which received the 0.03 per cent concentration. The pens on 0.02 and 0.01 per cent level of the drug suffered an 8 and 20 per cent mortality, respectively. In contrast, the mortality in the nonmedicated control birds was 44 per cent.

The data on the therapeutic value of the drug were difficult to interpret because of the variation among pens; also, the mortality in this phase of the study was scattered over such a long time that it was difficult to determine when enterohepatitis reached a stage that would be considered an outbreak under field conditions. A summary of the data is presented in table 1.

In part 2, the medication was started when the first enterohepatitis mortality occurred and continued for twenty-one days. The mortality was 10, 2, 32, 20, and 12 per cent on the drug levels of 0.05, 0.04, 0.03, 0.02, and 0.01 per cent, respectively. The mortality in the nonmedicated control group of this study was 6 per cent.

In part 3, the enterohepatitis mortality was 38 per cent in the group receiving the drug at a concentration of 0.1 per cent for fourteen days, 22 per cent in the group which received 0.1 per cent for seven days, 34 per cent in the birds given 0.1 per cent according to a three-three-three intermittent schedule, 36 per cent in those receiving 0.05 per cent for fourteen days, 32 per cent in the lot which received 0.05 per cent for seven days, 30 per cent in the lot which received 0.05 per cent according to a three-three-three intermittent schedule, and 24 per cent in the nonmedicated controls.

The data presented in table 1 indicate that none of the drug concentrations used affected body weight or feed efficiency significantly.

SUMMARY AND CONCLUSIONS

Data are presented on a study conducted under simulated field conditions to evaluate 2-amino-5-nitrothiazole medication in preventing and treating enterohepatitis (blackhead) in turkeys. Nine hundred and fifty birds separated into groups of 50 birds each were used in this test.

The data indicate that 2-amino-5-nitrothiazole, given continuously in an all-mash ration at the concentrations of 0.05, 0.04, and 0.03 per cent, was effective in preventing mortality from enterohepatitis. The concentrations of 0.02 and 0.01 per cent reduced mortality but were not sufficient to give a desired degree of protection.

Under the conditions of this experiment, 2-amino-5-nitrothiazole was apparently of little value in overcoming enterohepatitis when medication was started after the disease had broken out in a flock.

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Chemotherapy of Enterohepatitis of Turkeys
II. The Effect of 2-Amino-5-Nitrothiazole and 2-Acetylaminonitrothiazole on Egg Production Fertility, and Hatchability in Turkey Hens

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IT HAS BEEN shown that 2-amino-5-nitrothiazole and 2-acetylaminonitrothiazole are effective in the control of enterohepatitis in turkeys.[1, 2, 3] These drugs will probably be used in breeder flocks; thus, it was deemed advisable to determine their effects on egg production, fertility, hatchability, feed consumption, and body weight in laying turkey hens.

MATERIAL AND METHODS

The turkeys used in this study were selected by random sampling from a group of birds that were the survivors of a previous enterohepatitis experiment. They were housed in 16- by 16-ft. tents. The ground area of each tent was divided to make two 16- by 8-ft. pens in each tent.

Ten hens and 1 tom were placed in each pen. At the beginning of the experiment, 2 toms were placed in some pens, but fertility was found to be better when only 1 tom was present. Surplus toms were maintained apart, and toms were changed whenever loss of vigor or aggressiveness was observed. The hens were identified and weighed individually at the time they were placed in the pens. Because the toms were moved, their weights were not recorded.

A commercial all-mash turkey breeder ration was given to all birds. The drugs were mixed with the feed on the farm by a ½-ton electric feed mixer.

Weekly records of egg production, fertility, hatchability, and feed consumption were kept for each pen. Eggs were set weekly in a commercial turkey hatchery.

Feeding of the medicated mash began on December 29, 1950. The medication was continued for eighteen weeks; 2-acetylaminonitrothiazole was fed at concentrations of 0.1, 0.05, 0.03, and 0.02 per cent, and 2-amino-5-nitrothiazole at concentrations of 0.1, 0.05, and 0.03 per cent of the ration. Three pens, of 10 hens each, comprising one group were given each drug level. Seven pens containing 82 birds were maintained as nonmedicated controls.

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We are indebted to Merck and Company, Inc., Rahway, N. J., for the drugs and financial aid for this study.

DISCUSSION AND RESULTS

This study was conducted in the same tents and on the same ground where a high mortality from enterohepatitis had occurred in the previous experiment of which these birds were survivors. While designed to determine the effects of the drugs on laying turkey hens, some mortality from enterohepatitis was expected. Since only 1 bird died of this disease, enterohepatitis was not a significant factor. The absence of mortality apparently indicates that some degree of immunity developed from the previous Histomonas infection.

The results of this investigation are summarized in table 1, which records the per cent of egg production, fertility, hatchability, average feed consumption per bird for the entire eighteen weeks, and the initial and final body weights. Each group represents the average for three pens on any one level of medication, except group 8 which is an average of the seven control pens.

When subjected to statistical analysis, these data show that 0.1 per cent of the 2-amino-5-nitrothiazole did reduce egg production, fertility, and hatchability. The production, fertility, and hatchability was 32.9, 55, and 59 per cent, respectively, among the medicated birds; and 49, 72, and 72 per cent, respectively, among the controls.

The birds receiving the 0.1 per cent drug concentration consumed an average of 12.6 lb. less feed per bird and weighed an average of 1.8 lb. less per bird than the controls at the termination of the test. Statistical

analysis indicated that these variations in feed consumption and body weight may have been a chance variation. Since the drug at this level did reduce production, fertility, and hatchability, it is probable that the drug caused the reduction in feed consumption and body weights. Apparently, 2-amino-5-nitrothiazole at the 0.05 and 0.03 per cent drug concentrations did not affect egg production, fertility, hatchability, feed consumption, or body weights. While statistical analysis indicated that the fertility in the group on the 0.03 per cent drug concentration was significantly reduced, this was probably a chance variation, because the higher concentration of 0.05 per cent did not significantly reduce fertility.

TABLE 1—The Results of Feeding Laying Turkey Hens 2-Acetylaminonitrothiazole and 2-Amino-5-Nitrothiazole Continuously for Eighteen Weeks

Group No.	No. birds	Medication*	Production (%)	Fertility (%)	Fertile eggs hatched (%)	Ave. feed consumption per bird (lbs.)	Ave. weight per bird (lb.) Initial	Final
1	30	0.10% — #1	51.4	66.7	69.6	86.7	16.8	16.3
2	30	0.05% — #1	49.4	60.2	72.6	86.4	17.0	17.2
3	30	0.03% — #1	52.2	78.7	70.8	92.8	16.3	16.9
4	30	0.02% — #1	52.5	75.2	74.4	90.7	16.6	16.9
5	30	0.10% — #2	32.9	55.0	59.3	73.2	17.3	15.3
6	30	0.05% — #2	46.6	61.2	64.9	86.5	16.8	16.6
7	30	0.03% — #2	50.8	50.2	70.6	92.4	16.8	16.6
8	82	None	49.2	72.8	71.9	85.8	16.7	17.1

*#1 = 2-acetylaminonitrothiazole. #2 = 2-amino-5-nitrothiazole.

The data presented in table 1, when analyzed statistically, show that 2-acetylaminonitrothiazole at the concentrations used in this study did not affect production, fertility, hatchability, feed consumption, or weight gains.

While the results indicate some toxic effects after prolonged medication with 2-amino-5-nitrothiazole, it appears that this drug at a concentration of 0.1 per cent can be used in laying turkey hens for approximately two weeks without causing adverse results. The lower concentrations can be used for relatively longer periods without causing any marked reduction of feed consumption, production, fertility, or hatchability.

SUMMARY

Data are presented on the toxic effects of 2-amino-5-nitrothiazole and 2-acetylaminonitrothiazole when the drugs were given to laying turkey hens continuously in the ration for eighteen weeks. Each drug level studied was administered to 30 turkey hens separated into three pens of 10 birds each.

At a concentration of 0.1 per cent, 2-amino-5-nitrothiazole reduced egg production, fertility, and hatchability. A concentration of 0.05 or 0.03 per cent had no effect on egg production, fertility, or hatchability.

As used in this study, 2-acetylaminonitrothiazole apparently did not have toxic effects.

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Studies in Avian Neoplasia.
I. A Quantitative Evaluation of Neutralizing Antibodies for the Rous Sarcoma Virus in Avian Serums

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THE VARIATIONS in host-susceptibility and in the activity of different cell-free preparations of the Rous sarcoma virus make it difficult to obtain comparative quantitative evaluations of the neutralizing antibodies which may be present in avian serums. This report describes a procedure carried out in our laboratory to obtain such comparative data, which is based on information afforded by the findings of various investigators together with certain modifications.

This method involves the titration of tumor activity in Rous virus cell-free preparations by inoculating groups of chicks with fivefold serial dilutions to establish the minimal infective dose (m.i.d.). Mixtures of Rous virus and serums are also inoculated into other groups of chicks. An individual chick receives only one inoculum.* The antibody level, which is measured in neutralizing doses (n.d.) per milliliter of serum, is based on the evaluation of the tumor response after an observation period of thirty days.

LITERATURE

There is a recognized variation in the susceptibility of different breeds of chickens, as well as within the lines and families of a breed. Cole[13] described two selected lines with 12.5 and 77.2 per cent susceptibility, respectively, to the Jungherr sarcoma. Carr[6] developed a Brown Leghorn line (N-S) which was not susceptible to 10,000 infective doses of Rous virus, while the control line was uniformly susceptible. An inbred White Leghorn line with complete resistance to the Rous virus was described by Winton.[28] He also referred to lines which were quite susceptible to this agent. The activity of Rous virus depends on a number of factors, which include the age of the tumor at the time of harvest, type of virus diluent, and the temperature at which the virus preparations are incubated and stored. Carr[6] observed that the amount of infective agent present was inversely proportional to the age of the tumor. He found that the optimum age was fourteen to 22 days. According to Duran-Reynals,[17] more consistent tumor activity was obtained from the different tumor preparations through the harvest of a number of chicks of the same age after a uniform incubation period.

Mueller[23] found that the addition of cysteine hydrochloride to a filtrate reduced oxidative changes and protected the activity of Rous virus. Gye and Purdy[20] reported that the presence of HCN in the virus diluent reduced the loss of tumor activity due to oxidative changes. Similar results were obtained by Pirie,[24] who used cysteine and KCN-Ringer saline. Dmochowski[15] found KCN-Ringer saline quite satisfactory as a diluent for Rous virus.

The protective action of some avian serums was observed for this agent by Carrel.[11] Gye and Purdy[20] found that fowl, duck, rabbit, horse, goat, rat, mouse, and human serums reduced the rate of Rous virus inactivation, whereas guinea pig serum reduced the activity of this agent. Des Ligneris[14] considered guinea pig serum, heated for thirty minutes at 56 C., as protective. Gottschalk[19] found 2 per cent rabbit serum to be protective.

Carrel[11] reported that Rous virus was inactivated when incubated at 38 C. for fifteen hours. Sugiura and Benedict[26] found that Rous virus was unaffected when suspended in Locke-Ringer saline, pH 3.8 to 10.0 at 3 C. for twenty-four hours. Dmochowski[15] reported that cell-free

*Suggested by Dr. G. E. Cottral, veterinarian, of the Regional Poultry Research Laboratory, East Lansing.

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The photographs were taken by W. R. Bauriedel of the Veterinary Research Institute, Iowa State College, Ames.

From the Veterinary Research Institute, Iowa State College, Ames.

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Rous virus preparations held at 9 C. were less active than those prepared at 4 to 5 C. Duran-Reynals[17] observed that the preparation and storage of tumor tissue at reduced temperatures favored maintenance of activity. Bryan *et al.*[7] reported that neutral or slightly acid phosphate and borate buffers did not produce significant differences in the activity of Rous virus when incubated at 37 C. for three hours.

In order to measure the activity of Rous virus preparations, various methods have been developed. Murphy *et al.*[25] allowed the control tumors to reach a certain size before determining the relative activity of different preparations. Claude[12] utilized an index of activity, which depended on the size of the tumor and the number of takes. Dmochowski[15] employed a similar procedure. Serial dilutions of the Rous virus were inoculated by Carr[9] in order to establish a m.i.d. He found that 10^6 to 10^7 infective units were present in a gram of tumor tissue. Bryan[6] carried out quantitative studies of Rous virus activity by determining the latent-period response of chickens to subcutaneous injections. Duran-Reynals[17] referred to the measurement of activity in terms of an effective dose.

The qualitative evidence of Rous virus neutralization has been demonstrated by Carrel,[11] Fischer,[18] Andrewes,[2-5] Sittenfield *et al.*,[27] Des Ligneris,[14] McMaster *et al.*,[21] Amies,[1] Duran-Reynals,[16] and Carr.[10] In order to measure the amount of neutralizing antibodies present in serums, Duran-Reynals[17] employed serial serum dilutions. He inoculated four to six sites in each chick of a group with the different serum-virus mixtures, while one of these sites served as a tumor control. Duran-Reynals considered that evidence of neutralization was present if the tumor size were less than half the mean area of all the control tumors in the group.

MATERIALS AND METHODS

Rous Virus-Neutralization Test.—Preparation of the Tumor Suspension.—A desiccate* of the Rous sarcoma was suspended in saline and inoculated subcutaneously into 8 2-week-old White Leghorn chicks.** After twelve to fourteen days, the chicks were destroyed and neoplastic tissue from each of the inoculated chicks, free of blood and necrotic tissue, was collected in a covered sterile beaker partially immersed in water and ice. The pooled tumor tissue was suspended in sufficient KCN-Ringer saline, pH 7.2 to 7.4, to give a 10 per cent dilution.† The KCN-Ringer saline was prepared as follows:

NaCl	9.00 Gm.
N/10 KCN	40.00 ml.
N/10 HCL	32.00 ml.
KCl	0.30 Gm.
$CaCl_2$	0.25 Gm.
$NaHCO_3$	0.20 Gm.
Distilled water	1000.00 ml.

Tumor tissue and diluent were homogenized in a cold Waring blendor* for five minutes at 4 C., then stored at 4 C. for at least four hours. The homogenized tumor suspension was filtered through two layers of sterile gauze to obtain the filtrate which served as an inoculum to produce Rous tumors for harvest in twelve to fourteen days. After centrifugation** of the filtrate for thirty minutes, 4 C., at 8,000 to 10,000 revolutions per minute (relative centrifugal force of approximately 5,000x gravity) to remove cells, the supernatant was used as a source of Rous virus for the neutralization test and for the determination of the m.i.d. based on the lethal dose$_{50}$ (l.d.$_{50}$) according to the method of Reed and Muench.[26]

Technique of the Rous Virus-Neutralization Test.—The cell-free Rous virus suspension was diluted with KCN-Ringer saline solution containing 0.02 per cent of bovine albumin† to give a virus dilution of 1:25,000. The avian serums‡ to be tested were first diluted 1:2 with 0.9 per cent saline or used without dilution. If neutralizing properties are present and the information is desired, higher dilutions than 1:2 may be carried out later in fivefold steps. In some cases, serums were diluted 1:6,250 to obtain a neutralization end point. Serums may be stored in an electric or dry ice freeezer§ until needed. Duran-Reynals[17] found that storage at 4 C. for several months did not alter the neutralizing power of the serums. Equal volumes of the 1:25,000 virus dilution and 1:2 serum dilution were mixed together to give a total volume of 2 ml. and final dilutions of 1:50,000 and 1:4, respectively. The serum-virus mixtures were placed in the refrigerator, 4 C., overnight or for at least four hours. This method was used by Duran-Reynals,[16] who found that Rous virus inactivation occurred at ordinary room temperatures. He observed that incubation periods of four hours and twenty hours at 4 C. gave similar neutralization results. After incubation at 4 C., a

*Tumor desiccate was obtained from Dr. F. Duran-Reynals, Yale School of Medicine, New Haven, Conn., through Dr. G. E. Cottral, Regional Poultry Research Laboratory, East Lansing, Mich.

**Chicks were obtained from an inbred flock of White Leghorns maintained at the Veterinary Research Institute, Ames, Iowa.

†On the basis of tumor weight per volume of diluent.

*A. H. Thomas Co., Philadelphia, Pa., No. 4281-E.

**Refrigerated Centrifuge, Model PR-1, International Equipment Co., Boston, Mass.

†Crystallized bovine plasma albumin. Armour Laboratories, Chicago, Ill.

‡Routine samples collected from birds of the Veterinary Research Institute flock and from birds with regressed Rous tumors.

§Temperatures of about —20 C. and —70 C. were maintained in the electric and dry ice freezers, respectively.

0.2-ml. volume of the serum-virus mixture was inoculated subcutaneously, over the pectoral muscles of one side, into each of 5 2-week-old White Leghorn chicks.

The number of m.i.d.'s employed in this

Fig. 1—A 4+ Rous tumor produced in the chicken during an incubation period of 30 days.

study varied from 6 to 140, depending on the activity of the particular tumor harvest. The inoculum was placed subcutaneously in such a manner that no loss of the serum-virus mixture occurred through the site of needle entry. This was done by the direction of the needle point (24 gauge, 1.5 in.) through the skin and partially into the pectoral muscles and then back up next to the skin some distance from the site of entry. During the two- to three- hour period required for chick inoculations, serum-virus mixtures were kept in an ice water bath. The inoculated chicks were identified by wingbands and kept under observation thirty days for tumor development.

When no neutralization occurred, many of the chicks developed extensive Rous tumors within eighteen to twenty-four days and died. At the completion of the thirty-day observation period, all surviving chicks were destroyed and the extent of tumor development was evaluated. Duran-Reynals[17] employed an incubation period of thirty-two days.

Evaluation of Rous Tumors (*See* Fig. 1, 2, 3, 4).—Tumors were classified on the following basis: (a) 4+ referred to an extensive tumor growth involving the pectoral muscles on the inoculated side, which markedly distended the skin. The tumor growth usually contained a large amount of a hemorrhagic exudate with subcutaneous edema present over the abdominal muscles of the uninoculated side. Chicks affected with this type of tumor growth always died. (b) 3+ referred to a type of tumor development much like that in the 4+ group, except that distention of the skin was moderate in extent. This type of tumor usually developed into a 4+ response. (c) 2+ referred to a much less pronounced tumor development, essentially characterized by a hemorrhagic border along the periphery of the neoplastic tissue or a group of such tumors distributed over several square inches of the breast musculature. Usually, this type of tumor developed into a 3+ or 4+ response. (d) 1+ referred to a tumor without a hemorrhagic border. These tumors may enlarge to an inch or so in diameter, but usually regress without causing death. Many of these tumors are ¼ to ½ in.. in diameter and quite hard. (e) (—) No tumor development present.

Computation of the Tumor Index (t.i.).—The tumor response of the 5 inoculated chicks as a group was evaluated by the use of the t.i., in an attempt to determine if neutralization had occurred. If so, no tumor development was usually observed. However, certain serums with some neutralizing properties allowed only partial tumor development. The sum of the quantitative measurements of tumor development in each of the 5 chicks was divided by the number of observations to obtain the t.i. Values of 0.0 to 2.0 were considered as evidence that the serum being tested contained neutralizing antibodies. Tumor indexes of more than 2.0 were considered as indicative of no neutralization. An occasional chick was observed to be unusually susceptible to the Rous virus, as was suggested by the appearance of a tumor in the 1:31,250,000 dilution during

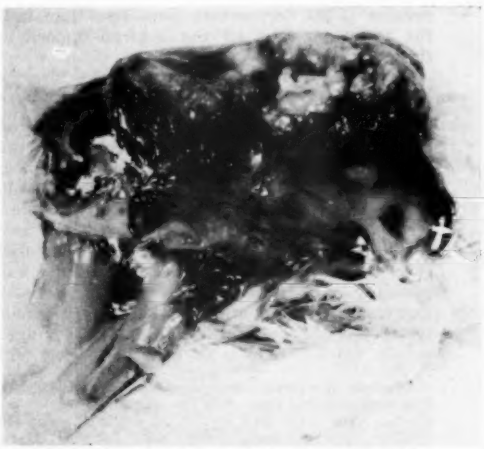

Fig. 2—A 3+ Rous tumor produced in the chicken during an incubation period of 30 days.

assay of tumor activity. Similarly, some chicks appeared quite resistant.

Determination of the Minimal Infective Dose.—The m.i.d., as employed in this study, referred to the greatest dilution of Rous virus capable of producing at least a 2+-type tumor within thirty days in 50 per cent of the inoculated chicks. The cell-free Rous virus suspension was diluted with KCN-Ringer saline containing 0.02 per cent bovine albumin, to give fivefold dilutions of 1 : 250,000, 1 : 1,250,000, 1 : 6,250,000, and 1 : 31,250,000. These dilutions were made up at the same time and treated in the same manner as the serum-neutralization tests and placed in the refrigerator overnight or for at least four hours. Each of the 5 to 7, 2-week-old chicks were inoculated subcutaneously on one side over the pectoral muscles with a 0.2-ml. quantity of a Rous virus

Fig. 3—A 2+ Rous tumor produced in the chicken during an incubation period of 30 days.

dilution. After an observation period of thirty days, the surviving chicks were destroyed and the extent of the tumor development was noted. Occasionally, a tumor developed in 1 of the chicks inoculated with the 1 : 31,250,000 virus dilution, while usually all of the chicks inoculated with the 1 : 250,000 dilution of virus developed tumors. The m.i.d. based on the $l.d._{50}$ end point varied with the different tumor preparations, but usually fell within the dilution range of 1 : 1,250,000 to 1 : 6,250,000.

Determination of Neutralizing Doses per Milliliter of Serum.—A n.d. of serum was the amount of serum necessary to prevent one m.i.d. from developing more than a 1+ tumor. This value was based on the m.i.d. of the particular virus preparation employed and the highest dilution of serum exhibiting definite neutralization. In the following example, a 1 : 4 dilution of serum exhibited neutralization of a 1 : 50,000 dilution of virus in which the m.i.d. was present in the 1 : 6,250,000 dilution.

Fig. 4—A 1+ Rous tumor produced in the chicken during an incubation period of 30 days.

The final dilution of the Rous virus employed in this test was 1 : 50,000. The factor of one-half was used, since the m.i.d. was based on a 0.2-ml. quantity of virus; whereas, the n.d. was based on a 0.2-ml. quantity of equal parts of virus and serum. Since 0.1 ml. of serum was considered to be contained in the inoculum in the neutralization test, the results were multiplied by ten for a 1-ml. quantity. The factor of four referred to the dilution of serum used in this test. The n.d.'s were calculated from the following:

$$\frac{6{,}250{,}000}{50{,}000} \times \tfrac{1}{2} \times 10 \times 4 = 2{,}500 \text{ n.d.'s per ml. of serum.}$$

Results and Discussion

Figures 1, 2, 3, and 4 represent the different types of Rous tumors and the respective quantitative evaluations of neoplastic development employed in obtaining the t.i.'s.

Table 1 shows the host-variability to the Rous agent, as observed with stock tested at this laboratory.

Table 2 demonstrates the effect of temperature and the type of diluent on the activity of the Rous virus.

Table 3 lists the results of neutralization

tests carried out with various serums under different conditions.

The high susceptibility to Rous virus of the White Leghorn chicks at this laboratory prevented the use of more than one inoculum in a single chick. Difficulties arose in the interpretation of results when more than one inoculum was used. This may be readily visualized when the extent of tumor formation due to only a single inoculum is observed (fig. 1 and 2). The localized tumors described by Duran-Reynals[17] were only observed in some cases (fig. 3 and 4). According to Des Ligneris,[14] the faster growing tumor in a bird may have an immunizing effect on a slower growing tumor at another site. Carr[8] claimed that saline-inoculated control sites may develop tumors when the tumor agent has been given into the breast of the same day-old-chick. Bryan[6] found that biological responses to the inoculation of Rous virus into the thigh regions of some chicks may differ significantly from those of the wing and breast regions.

TABLE 1—Variation in Susceptibility of White Leghorn Families and of Other Breeds of Chickens to Rous Sarcoma Virus

Family	No. tested	Tumor Index‡
A	7	1.30
B	4	0.75
C	8	1.10
D	5	0.60
E	7	0.45
F	5	2.80
G	5	1.40
K	3	4.00
L-1	3	1.33
L-2	3	4.00
M	7	1.00
N	3	1.33
O	3	3.33
V*	27	2.00
W**	13	3.30
Z†	13	0.00

*Rhode Island Red general population. **White Leghorn general population. †Egyptian breed obtained from Dr. A. W. Nordskog, Poultry Department, Iowa State College, Ames. ‡All these chicks were inoculated with 57 minimal infective doses of Rous virus.

The t.i.'s listed in table 1 demonstrate the differences in susceptibility to Rous virus between families of a breed as well as between breeds. If comparable results are anticipated, it is certainly important to select chicks from families and lines which are uniformly susceptible. Such a susceptible population may be selected through the methods of Cole[13] and Carr.[8]

In table 2, trial 1, KCN-Ringer diluent protects the activity of Rous virus at 37 C., while a much lower virus dilution in saline lost its activity. Even the addition of 0.02 per cent albumin did not give complete protection. The protective effect of albumin for Rous virus is shown rather definitely by trials 2 and 3 at 37 C. and 4 C., respectively. Some protective action is demonstrated by albumin in trial 4 at 4 C.

TABLE 2—Relation of Diluent and Incubation Temperature to the Activity of Rous Virus

Trial	Date run	Diluent	Temperature*	Virus dilution	Tumor index
1	2–17–50	Saline**	4 C.—3 hr.	1 : 450,000	4.0
		KCN-Ringer	4 C.—3 hr.	1 : 450,000	4.0
		Saline	37 C.—2 hr.	1 : 60,000	0.0
		KCN-Ringer	37 C.—2 hr.	1 : 450,000	4.0
		Saline + alb.†	37 C.—2 hr.	1 : 450,000	2.5
2	3–9–50	Saline + alb.	37 C.—2 hr.	1 : 640,000	2.6
		Saline	37 C.—2 hr.	1 : 120,000	3.2‡
3	3–9–50	Saline	4 C.—3 hr.	1 : 240,000	2.4
		Saline + alb.	4 C.—3 hr.	1 : 640,000	4.0
4	3–16–50	Saline	4 C.—3 hr.	1 : 1,280,000	2.4
		Saline + alb.	4 C.—3 hr.	1 : 1,280,000	3.2
5	4–13–50	KCN-Ringer + alb.	4 C.—20 hr.	1 : 5,120,000	3.2
		KCN-Ringer + alb.	37 C.—1 hr.	1 : 5,120,000	2.6
		KCN-Ringer + alb.	4 C.—3 hr.	1 : 5,120,000	3.4
6	5–19–50	Saline	4 C.	1 : 5,120,000	2.8
		Saline	4 C.—20 hr.	1 : 5,120,000	2.2

*All these preparations were immersed in an ice water bath for two to three hours while the inoculations were being carried out. **Refers to 0.9 per cent NaCl solution. †Bovine albumin added to give a 0.02 per cent concentration. ‡A tumor index of 0.0 was observed for each of the following virus dilutions: 1 : 240,000, 1 : 320,000, 1 : 480,000, and 1 : 640,000.

Trial 5 demonstrates the protective effect of KCN-Ringer and albumin, even when rather high virus dilutions are tested. The crystallized bovine albumin affords a uniform source of protein, which gives the at 37 C. for one hour, the neutralization results are quite comparable to those obtained by incubation overnight at 4 C.

When serums 130LI, 356aI, and 356I were heated at 56 C. for thirty minutes to

TABLE 3—Rous Virus-Neutralization Tests

Serum	Date tested	Serum dilutions and tumor indexes 1:50	1:250	1:1,250	n.r.*	m.l.d.** inoculated	n.d. ml. serum†	
53T	5-26-50	0.0	0.0	3.2	+	6.4	16,000	....
75C	6-2-50	2.8	4.0	3.4	—	72.4	....	....
88V	6-2-50	0.2	2.4	3.0	+	72.4	36,200	....
89V	6-2-50	1.2	2.4	4.0	+	72.4	36,200	....
130L	6-2-50	0.0	0.0	0.0	+	72.4	905,000	....
130L	7-13-50	0.0	0.0	0.0	+	12.5	15,625	20 hr.—4 C.
130LI‡	7-13-50	0.8	0.0	0.0	+	12.5	15,625	....
311	5-11-50	4.0	4.0	3.6	—	32.0	....	....
311	5-26-50	2.2	4.0	4.0	—	6.4	....	....
339	5-11-50	0.0	0.4	0.0	+	32.0	40,000	....
339	6-2-50	0.0	0.0	0.0§	+	72.4	4,525,000	....
356	5-5-50	0.0	0.0	0.0	+	78.5	98,125	20 hr.—4 C.
356a	5-19-50	0.0	0.0	0.0	+	51.2	64,000	....
356aI	5-19-50	0.0	0.0	0.0	+	51.2	64,000	....
356b	6-30-50	0.0	0.0	0.0	+	25.0	31,250	20 hr.—4 C.
356I	5-5-50	0.0	0.0	0.0	+	78.5	98,125	20 hr.—4 C.
1¶	4-6-50	0.0	0.8	3.4	+	16.0	4,100	1 hr.—37 C.
1¶	4-6-50	0.0	0.0	2.8	+	16.0	4,100	20 hr.—4 C.
2¶	4-6-50	0.0	1.6	2.6	+	16.0	4,100	1 hr.—37 C.
2¶	4-6-50	0.0	1.6	3.0	+	16.0	4,100	20 hr.—4 C.
3¶	4-6-50	1.0	1.6	3.75	+	16.0	4,100	1 hr.—37 C.
3¶	4-6-50	0.0	0.0	3.0	+	16.0	4,100	20 hr.—4 C.
		1:2	1:4					
S13	3-21-51	0.0	...	...	+	140.0	280	....
S13	3-21-51	0.0	...	...	+	140.0	280	....
598	1-23-51	...	4.0	...	—	....	....	....
598	1-23-51	...	4.0	...	—	....	....	....

*Neutralization reaction. Considered positive if tumor index was 2.0 or less. **Minimal infective doses. †Neutralizing doses per milliliter of serum. ‡The letter "I" after a serum number indicates that alexin inactivation was carried out in a water bath for thirty minutes at 56 C. §At 1:6,250 serum dilution, the tumor index was 1.6. ¶These serums were diluted 1:20, 1:100, and 1:500.

Rous virus protection more comparable to that offered by avian serum proteins present in the serum-neutralization tests. Higher dilutions of Rous virus in trial 6 may have shown more loss of activity after incubation in saline at 4 C. for twenty hours.

Avian serums were tested under identical conditions, as well as under different conditions, to determine if comparable results would be obtained. In table 3, serums 130L, 311, 339, and 356 were retested at different times to show that similar results were obtained. When serums S13 and 598 were retested under identical conditions, the results were found to be quite comparable. No inconsistencies in the results have been observed during the testing of over 1,000 avian serums for Rous-neutralizing antibodies.

Apparently, when serum-virus mixtures (serums, 1, 2, and 3, table 3) are incubated destroy complement, no effect on the neutralizing capacity was observed. Such results were expected, since Des Ligneris[14] found that complement had no adverse effect on the Rous virus.

SUMMARY

A method for evaluating the quantity of Rous-neutralizing antibodies present in avian serums is described. No inconsistencies were observed in testing over 1,000 serums.

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Morphogenesis of the Thymus of Chicken Embryos

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Literature

Verdun[22] studied the early development of the thymus in the chick and stated that the third pair of clefts were not open to the exterior after 124 hours of incubation. This author stated further that their internal and external extremities atrophied early, while the dorsal intermediate portions formed an entodermal epithelial pouch against the internal jugular vein on either side of the neck. These pouches then gave rise to an epithelial cord on each side of the neck and extended the full length of the jugular vein, corresponding to thymus III.

This author also found that the fourth pair of clefts separated from the pharynx at 164 hours of incubation. These clefts were found to give rise, through the dorsal and superior region, to a thymic bud on either side of the neck, thymus IV. It was found that eventually thymus IV terminated by fusion with thymus III.

Lillie[10] concluded that the thymus of the chicken embryo had a double origin on either side of the neck. The principal portion of the thymus, which Lillie called thymus III, was thought to be derived from the dorsal wall of the intermediate part of the third visceral pouch, then elongated to form an epithelial cord along the jugular vein. This author believed that a smaller portion, thymus IV, was derived from a corresponding part of the fourth visceral pouch and fused with thymus III. These observations agreed with those of Verdun.[22]

A more complete study of the development of the thymus has been made in other bird species. Helgesson[8] studied the development in *Passer domesticus* up to the 14-mm. embryo. Hamilton[7] studied the development in *Anas boschas* up to the 45-mm. embryo. For a study of comparative morphogenesis of the thymus in mammals, the reader is referred to the work of Godwin.[4]

Experimental work on the thymus of the chicken showed that both thymectomy and feeding of thymic tissue gave contradictory and inconclusive results.[1, 2, 11, 12, 13, 15]

For a comparative study of the morphogenesis of the thymus with the thyroid and parathyroid glands, the reader is referred to the reports of Venzke.[20, 21]

Greenwood and Blyth[6] frequently observed thymic tissue near and posterior to the thyroid. The distribution of the thymic tissue was variable; it was present under the thyroid capsule penetrating the substance of the thyroid gland, external to the thyroid capsule, or extending around the posterior lobe of the thyroid and coming to lie between it and the parathyroid glands. Terni[17, 18] reported "thymus-like tissue" within the substance of the parathyroid glands of the fowl.

Dantschakoff[3] assumed that, in the bird, the reticulum and Hassall's corpuscle were the only elements which developed from the original epithelial anlage. This author was of the opinion that the small thymic cells (or lymphocytes) and eosinophils were of mesenchymal origin.

Sun[16] stated that, prior to fifteen days of incubation, the thymus of the chick appeared as a group of undifferentiated cell masses held together by mesenchymal tissue. The cortex, medulla, and Hassall's corpuscles did not definitely show until fifteen days of incubation.

Maughan[11] stated that there were, in all, approximately 14 distinct thymic lobes, usually seven on each side of the neck, loosely joined together. No thymic material was found in the thoracic cavity.

Pensa[14] described myoid or sarcolytic cells in the thymus of birds and reptiles. This author also believed that these cells were embryonic muscle fibers which resulted by inclusion from the musculature of the branchial clefts.

Latimer[9] found that, at first, the thymus formed a little less than 0.3 per cent of the net body weight of the single-comb White Leghorn chick. This study showed that the thymus increased both in percentage and absolute weight up to sexual maturity, and then underwent involution. The thymic changes were found to be more closely related to age than to body weight. Venzke[19] reported on the thymic growth rate in both sexes of chicken embryos.

Greenwood[5] observed a definite relationship between the gonad and thymus involution in the fowl.

Materials and Methods

All embryos and chickens used in this study were of a pure single-comb White Leghorn stock maintained by the Department of Poultry

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Sincere thanks are extended to Dr. H. L. Foust, Department of Veterinary Anatomy, Iowa State College, Ames, for his counsel; to Dr. M. Lois Calhoun, Department of Veterinary Anatomy, Michigan State College, East Lansing, for her assistance in preparing photomicrographs.

Husbandry at Iowa State College, Ames. Throughout the study, the fertile eggs were obtained from the same group of hens.

A series of 420 embryos, ranging from ninety-six hours of incubation to hatching, was used for the morphological studies. The material for study of the thymus was obtained by serially sectioning the cervicothoracic region at intervals of approximately twelve hours.

The tissues were fixed in Bouin's or Helly's fluid and sectioned in Altman's paraffin mixture at 6 to 8μ in thickness. Those tissues fixed in Bouin's fluid were stained with hematoxylin and eosin. Those fixed in Helly's fluid were stained with Mayer's hematoxylin and azure II eosin or Mallory's connective tissue stain.

Helly's fluid, which was the most satisfactory, did not destroy the basophilic character of the cytoplasm of the lymphocytes and showed clearly the red blood corpuscles in the thymic tissue.

OBSERVATIONS

The greatest development of the visceral arches occurs between ninety-six and 110 hours of development. The second visceral arch, which is the largest, overlaps both the first and third. All of the arches are wedge-shaped. The fourth visceral arch is small and incomplete ventrally.

By the termination of the fourth day, the third visceral pouch has formed a small, round, dorsal cleft and a long, ventral, fissure-like cleft. These close at approximately 120 hours. The fourth visceral pouch connects with ectoderm at its dorsal end at approximately ninety-six hours, but no cleft develops.

At 120 hours of development, the second, third, and fourth visceral pouches continue to retain their connections with the corresponding ectodermal grooves. As a result, the visceral pouches and ectodermal furrows are drawn into rather long epithelial tubes, as growth continues. This arrangement causes the original closing plate to become deeply invaginated.

It is now possible to observe the double origin of the thymus (fig. 1). The large portion, or thymus III, is derived from the ventral and lateral wall of the third visceral pouch. This mass of epithelial cells elongates to form an epithelial cord extending along the jugular vein. Thymus IV, a smaller portion than thymus III, is derived from the dorsal wall of the intermediate part of the fourth visceral pouch, and fuses with thymus III. The floor of the fourth visceral pouch develops a posteroventral evagination at 120 hours; this eventually becomes the ultimobranchial body. The dorsal aspect of the third visceral pouch disappears.

The third and fourth visceral pouches sever their connections with the pharynx at 134 hours and remain as patches of epithelium in the mesenchyma of the neck, adjacent to the jugular vein. The ventral portion of the epithelium from the third pouch has fused with the dorsal portion from the fourth to form a thymus body on each side of the neck.

By 134 hours, the ultimobranchial body is completely separated from the main part of the fourth pouch.

The thymus at 165 hours is an epithelial structure whose appearance is that of an elongated mass of protoplasm and syncytial in character. Deeply stained, finely branching protoplasmic threads give the syncytial mass a distinct reticular appearance.

The mesenchyma closely invests the thymus and, in places, cytoplasmic processes of the mesenchymal cells appear to fuse with the cytoplasm of the epithelial cells. Large lymphocytes are found scattered throughout the mesenchyma, and local accumulations appear near large blood vessels (fig. 2). The lymphocytes are characterized by a basophilic, nongranular cytoplasm and a large, highly chromatic nucleus. No lymphocytes have yet appeared in the thymic tissue. However at 177 hours, an occasional large lymphocyte is noted to have migrated into the periphery of the thymus from the surrounding mesenchyma.

At 206 hours, the thymic tissue shows an increase in large lymphocytes and has migrated posteriorly into contact with the thyroid glands.

At 213 hours, irregular lobules appear as a result of growth of the epithelial cells. Lobule formation begins near the head and disappears in the midcervical and thoracic regions. The largest number of lymphocytes are found in the tissue nearest the head. The surrounding mesenchyma also shows an increase in lymphocyte content. The thymic epithelial cells are characterized by relatively light-staining cytoplasm and large, round to oval, sparsely chromatic nuclei. Blood vessels have not invaded the thymic tissue.

In the midcervical and thoracic regions at 228 hours, the thymic tissue has become lobulated and more lymphocytes have appeared. The lymphocytes located in the

mesenchyma surrounding the thymus are of both the large and small type. The small lymphocytes are characterized by a rather small, heavily chromatic nucleus, surrounded by a thin layer of cytoplasm. These increase near the periphery, but they predominate the entire field. The epithelial or reticular cells are highly variable in shape and size. These cells are so closely packed in most of the tissue that it is difficult to

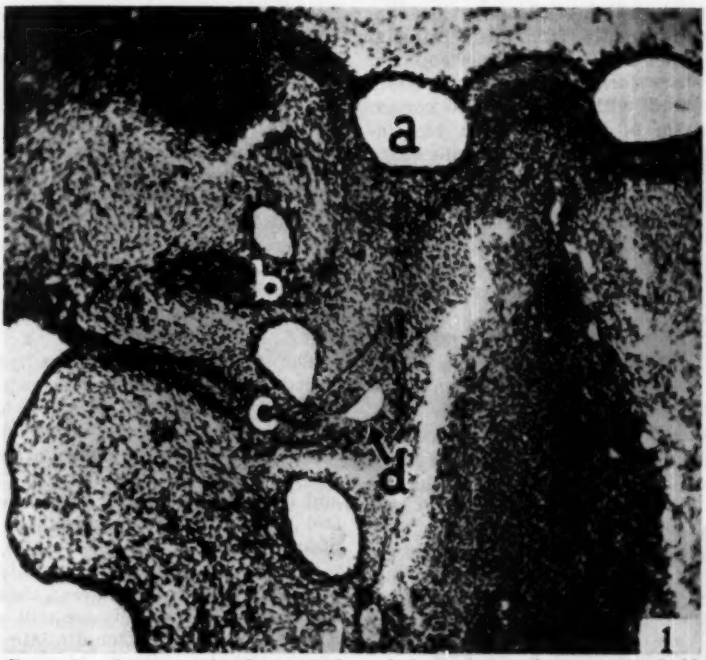


Fig. 1—The section of tissue is taken from a 120-hour chicken embryo and presents the double origin of the thymus from visceral pouch III and IV. The small evagination from the posterior and ventral floor of visceral pouch IV becomes the ultimobranchial body. Hematoxylin-eosin stain. x 100. a = pharynx; b = visceral pouch III; c = visceral pouch IV; and, d = evagination which becomes the ultimobranchial body.

cells first appear in the thymic tissue at 240 hours.

Lobule formation continues at 252 hours, but is poorly developed in the thoracic region. A layer of mesenchymal cells closely invests the outer surface of the lobes, forming a limiting membrane for the outer surface of the thymus. Blood vessels are numerous in the interlobular septa, but have not invaded the lobules.

At 260 hours, the periphery of the thymus in some portions shows an epithelial syncytium. Occasionally, a vessel of a capillary nature can be found in the thymic lobules.

The number of lymphocytes is markedly reduced within the thymus at 272 and 284 hours (fig. 3). Epithelial cells continue to make out the cell outlines distinctly.

At 295 hours, the blood supply to the thymus has increased noticeably. Again, rather large numbers of lymphocytes of various sizes are appearing in the thymic tissue, especially around blood vessels within the tissue and in the interlobular connective tissue. The small thymic cells or small lymphocytes are rather prominent, and are scattered throughout the glandular tissue.

The tissue within the central part of the gland lobules is loosely arranged, while the periphery is rather compact. The vascular supply is greatest in the central portion of the lobule, where fewer lymphocytes are present and where the cells are mostly large epithelial cells similar to those found early in the thymic tissue. The striking feature

about these reticular cells is that they have an extremely large nucleus, one nucleolus, and almost no chromatin.

Thymic lobules at 319 hours possess well-developed cortical and medullary zones, but these two zones can not be distinctly separated from one another (fig. 4). The medulla formed directly from the epithelial syncytium, whose cells have hypertrophied. The nuclei are large and relatively clear when compared with those of the cortex. The cytoplasm of the syncytium has increased in amount. The cytoplasmic processes of the medulla appear to be continuous with those of the cortex. The epithelial cells in the medulla occur singly or in groups of two or three. These groups of cells are perhaps the beginning of Hassall's corpuscles, since their nuclei are markedly enlarged.

All types of lymphocytes are found in the cortex and scattered in the meshes of the reticulum of the medulla. The reticulum of the cortex is also formed from the cytoplasmic syncytium of the epithelial cells.

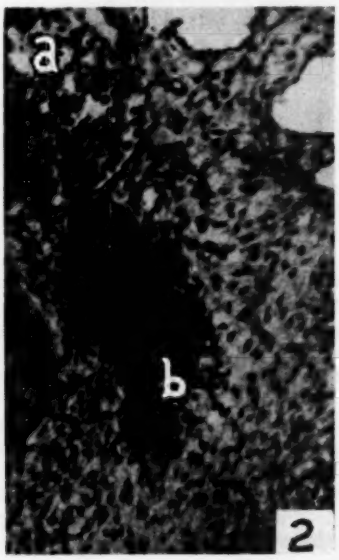
At 345 hours, the medulla contains many lymphocytes, which make it difficult to sharply distinguish the cortex. Hassall's corpuscles are still in the process of development.

The distinctness of the cortical and medullary zones of the thymic lobules can be beautifully demonstrated at 357 hours. Very few lymphocytes remain in the medulla. Small thymic cells are the predominating type in the cortex, but intermediate and large lymphocytes are present. The reticulum is much finer in both the cortex and medulla. The vascularity of the cortical zone is increasing; however, most large vessels are harbored in the interlobular connective tissue.

At 375 hours, the lobules of the thymus are closely packed, and the cortex is increasing in thickness (fig. 5). Small lymphocytes are by far the most numerous, yet large ones are still plentiful in all parts of the cortex.

The medulla contains less lymphocytes than the cortex. Where the medulla of the lobules joins the central stem, it contacts the deep portion of the interlobular septa. The strands of the reticulum in the medulla are much coarser than those of the cortex, and its epithelial nuclei are larger, clearer, and more completely surrounded by cytoplasm.

The interlobular connective tissue septa are decreasing in thickness; those lodging the larger blood vessels are still very wide. Fibers of the septa often dip into the cortex of the lobules. Small blood vessels are



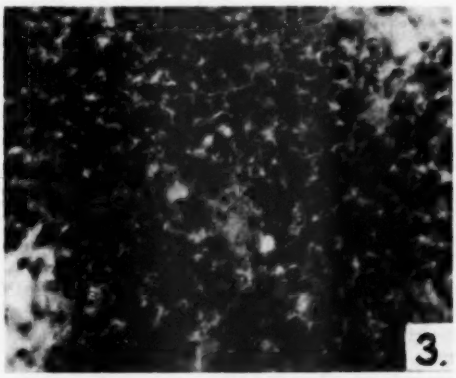


Fig. 2—A section of tissue taken from the cephalic region of the neck in a 165-hour chicken embryo. Observe the large number of lymphocytes outside the large blood vessel in the surrounding mesenchyma. These lymphocytes, at a later period, migrate into the thymic primordia. Hematoxylin-eosin stain. x 200. a = blood vessel; and b = lymphocytes.

Fig. 3—A section of thymic tissue taken from a 284-hour chicken embryo before a definite cortex and medulla have formed. Hematoxylin-eosin stain. x 200.

found throughout the septa and often enter the cortex. Few lymphocytes appear in the septa.

One specimen at 388 hours, exhibited a portion of the thymus and ultimobranchial body within the thyroid tissue. The ultimobranchial body does not change into thyroid tissue, nor does it develop follicles characteristic of the thyroid gland.

At 400 hours, eosinophil cells appear in the thymus in appreciable numbers. The interlobular septa contain numerous lymphocytes, red blood cells, and eosinophil cells, both singly and in groups. The largest number of eosinophil cells is found in the medulla. Those in the cortex are widely scattered and usually occur singly. The number of eosinophils appears to be evenly divided throughout the various aggregations of thymic tissue (fig. 6).

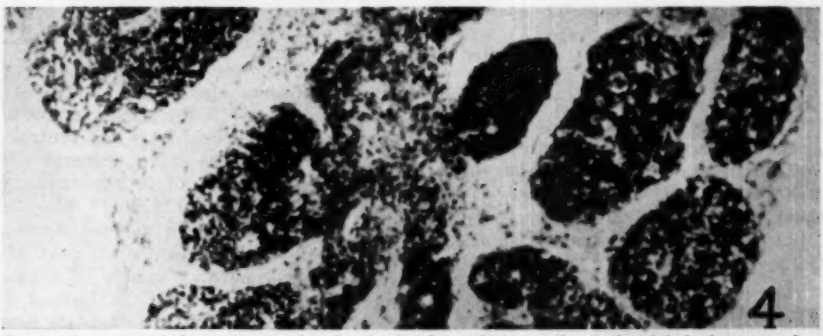


Fig. 4—A section of thymic tissue taken from a 319-hour chicken embryo after lobule formation has occurred. Hematoxylin-eosin stain. x 200.

At 412 hours, most of the thymic lobules contain groups of erythrocytes, of which the largest numbers occur nearest the head. Erythrocytes are distributed in both the cortical and medullary zones.

By 426 hours, many different cells types have appeared in the thymus. Perhaps at this time it would be well to present a detailed description. The connective tissue ingrowths from the primitive thymic capsule produce the primary lobules. These ingrowths of connective tissue represent the beginning of thymic stroma. The lobules are widely separated by loose cellular connective tissue but, as the rate of growth of the thymus increases, the lobules become closer and, at the present time, the stroma is compressed into thin septa. The stroma, in addition to connective tissue cells, contains lymphocytes in small numbers, reticular cells, an occasional esosinophil cell, and the large blood vessels.

The small thymic cells at present are found closely aggregated in the cortex. The few which are found in the medulla lie in the meshwork of the reticulum.

The original epithelial cells of the thymus, or the present reticular cells, are variable in size and shape, and are two or three times the size of the small thymic cells. Their outlines are indefinite. The protoplasm is drawn out into irregular prolongations which anastomose with similar processes from other reticular cells, producing a syncytium. Mitoic activity is common among these cells. The reticular cells are most easily demonstrated in the medulla. Occasionally, some of these cells in the medulla become markedly hyperthrophied and include groups of three or four cells. These groups have the appearance of Hassall's corpuscles and may contain degenerate small thymic cells.

Few eosinophil cells appear in the thymus at this time. Many erythroblasts are present and are characterized by a faint brick-red cytoplasm, a very granular nucleus, and are found singly and in groups. An occasional multinucleated giant cell is found.

A large space is occasionally found within the central part of that portion of the thymus nearest the thyroid. These spaces are lined with a simple squamous epithelium. When traced through serial sections, these spaces end blindly or as a solid epithelial cell nest, and usually appear to be continuous with the reticulum.

At 438 hours, there is a marked increase in eosinophils and hypertrophied reticular cells in the medulla.

At 450 hours of incubation, the connective tissue capsule surrounding each lobe of the thymus has become well developed. Thin connective tissue septa, which have become compressed by the growth of the parenchyma, are detached from the capsule. These interlobular septa do not penetrate the gland substance except for the thin connective tissue sheath accompanying the small blood vessels. The cortex is composed almost entirely of small thymic cells. The medulla consists predominantly of reticular cells, many of which are smaller than those found in the cortex.

The medulla also contains the bodies of Hassall. These structures are round in outline, acidophilic in staining, and measure from 30 to 40μ in diameter. They are composed of concentrically arranged cells, most of which show evidences of degeneration. Reticular cells are connected at the periphery of each Hassall's body.

At 462 hours, many mitotic divisions are occurring, especially at the junction of the medullary and cortical portions. The cortex and medulla are densely packed with cells. Eosinophils have increased, especially in the medulla, and Hassall's corpuscles have decreased.

Fig. 5—A section of thymic tissues taken from a 375-hour chicken embryo. Hematoxylin-eosin stain. x 200.

The thymus remains unchanged, except for growth, at 474 and 498 hours (fig. 7). One embryo was observed in which thymic tissue surrounded most of the anterior lobe of the parathyroid. Another embryo was studied in which the thyroid was completely surrounded by thymic tissue.

DISCUSSION

After 120 hours of incubation, it is possible to observe the double origin of the thymus (fig. 1). The large portion, or thymus III, is derived from the ventral and lateral wall of the third visceral pouch. This observation is not in agreement with that of Verdun[22] and Lillie,[10] who found that thymus III was derived from the dorsal wall of the intermediate part of the third visceral pouch. Thymus IV, a smaller portion than thymus III, is derived from the dorsal wall of the intermeidate part of the fourth visceral pouch, and eventually fuses with thymus III. This observation has been made previously by Verdun[22] and Lillie.[10]

The third and fourth visceral pouches sever their connections with the pharynx by 134 hours of incubation. Verdun[22] observed that the fourth cleft was separated from the pharynx at 164 hours of incubation.

At 165 hours, large lymphocytes are scattered here and there throughout the mesenchyma, and especially around the blood vessels of the neck and upper thoracic regions (fig. 2). After 177 hours, an occasional large lymphocyte migrates into the periphery of the epithelial thymic tissue from the surrounding mesenchyma.

Irregular lobules form in the thymus as a result of growth of the epithelial cells (fig. 4). Connective tissue ingrowth from the primitive capsule, coupled with growth and increase in the number of epithelial cells, produces the primary lobules. The ingrowths of connective tissue from the capsule mark the beginning of the thymic stroma. The lobules are widely separated in early embryonic life by loosely arranged cellular connective tissue. As the rate of growth of the thymus increases, the lobules become intimately associated and compress

the connective tissue stroma into thin septa late in embryonic life. The interlobular septa, as a rule, do not penetrate the parenchyma except for thin strands accompanying blood vessels. All blood vessels interlobular septa. It is concluded from this that the lymphocytes, which are present in the surrounding mesenchyma of the thymus, migrate into the tymic tissue along the interlobular septa. Since there are no

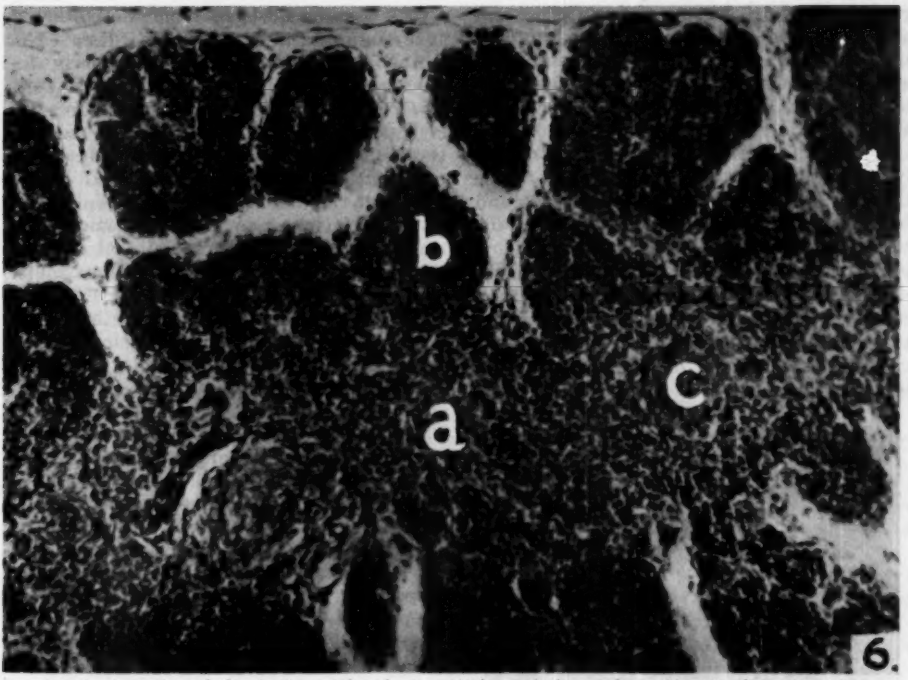


Fig. 6—A section of thymic tissue taken from a 400-hour chicken embryo. Hematoxylin-eosin stain. x 200. a = medulla of thymic lobule; b = cortex of thymic lobule; and c = beginning formation of Hassall's corpuscle.

are contained in the stroma. Many cellular elements are always present in greater or less numbers in this stroma. The formation of lobules begins after 213 hours of incubation at the cephalic end of the thymus. It is not until after 228 hours that the thymic lobules form in the midcervical and thoracic regions. At this period, there are no blood vessels in the thymus. Small thymic cells are beginning to appear in the mesenchyma surrounding the thymic anlage.

After 252 hours, both large and small lymphocytes are rather prevalent in the thymic primordium. Blood vessels are numerous in the interlobular septa but do not appear in the thymic parenchyma. Many mitotic figures are present among the large lymphocytes. At this time, the largest number of lymphocytes are found in the transitional forms between the epithelial cells and lymphocytes, and since blood vessels are absent in the thymic anlage, it is concluded that the first source of lymphocytes in the thymus is a result of their migration from the surrounding mesenchyma.

The small lymphocytes arise from the cellular division of the large lymphocytes. This fact is based on the following: there are no blood vessels present in the thymic parenchyma at this period; transitional cell forms between epithelial cells and lymphocytes are absent; the lymphocyte series consists of large, intermediate, and small stages; and frequently mitosis is present among these lymphocytes.

Between 260 and 295 hours, there is a marked decrease in the number of lympho-

cytes in the thymus. During this time, however, small blood vessels appear in the thymic parenchmya, and an epithelial syncytium forms about the periphery. Also during this period, embryos may be observed in which the thymic tissue has grown posteriorly between the jugular vein and the thyroid, terminating in the space between the posterior pole of the thyroid and the parathyroid glands. This had previously been observed by Greenwood and Blyth[6] in adult chickens.

After 295 hours, large numbers of the lymphocytic series are again present in the thymic parenchyma, particularly around blood vessels near the periphery, and in the interlobular connective tissue. The periphery is rather compact as contrasted with the central portion of the lobules, which is composed primarily of loosely arranged epithelial cells. This is followed by an increase in the number of small thymic cells in the periphery.

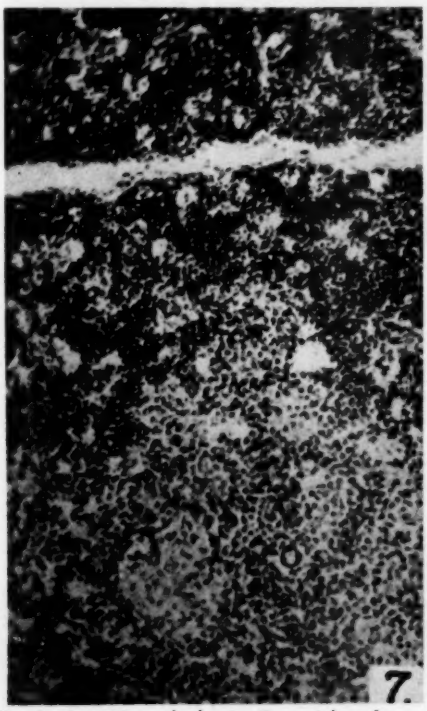


Fig. 7—A section of thymic tissue taken from a 474-hour chicken embryo. Hematoxylin-eosin stain. x 200.

After 319 hours, well-developed cortical and medullary zones are distinguishable (fig. 4), but it is not until after 357 hours that a distinct and definite separation of the two zones can be made (fig. 6, 7). Sun[16] made a similar observation.

The medulla develops from the epithelial syncytium of the thymic primordium. The epithelial or reticular cells are clearly demonstrable in the cortex at 345 hours. Very few lymphocytes are present in the medullary zone when both the cortex and medulla become definite. Development of the cortical reticulum is gradual. Prior to 357 hours, the syncytial network in the medulla and cortex is coarse. After the cortical and medullary zones become definite, the coarse syncytial network gradually becomes a fine reticulum.

After 319 hours, a few of the epithelial cells are organized into groups of two or three which have extremely large nuclei. These groups of cells mark the beginning of the formation of Hassall's corpuscles. These bodies vary in size and number as embryonic growth progresses. At 450 hours, the typical Hassall's corpuscles have diameters varying between 30 and 40μ (fig. 6).

As the medulla becomes well developed, the number of lymphocytes within this zone is less (fig. 7). However, all three sizes of lymphocytes are present in the medulla throughout its development. The apparent disappearance of lymphocytes from the medulla, as development progresses, may be attributed to one of the following conditions. The lymphocytes may migrate from the medulla into the cortex. The increase in volume of the medulla, produced by the increase in epithelial cells, may spread the lymphocytes of the medulla over a larger area. Thus, there may be a scattering but no decrease in the number of lymphocytes. The third interpretation is made on the basis of the blood supply to the thymus. The arteries are distributed in the cortical zone of the lobules through the cortical stroma. In this area, the arteries form into a capillary network which converges toward the venous vessels of the medulla. On the basis of this vascular arrangement, it may be possible for the lymphocytes to migrate from the cortex to the medulla where they enter the venous capillaries and are carried into the general circulation.

After 400 hours, free erythrocytes and

eosinophils are present in rather large numbers. It is not until late embryonic life that the eosinophils become numerous. There may be a correlation between the presence of free erythrocytes and eosinophils, since they never occur alone. The eosinophil cells, in general, are more numerous in the connective tissue of the interlobular septa than in the lobules of the thymus. Within the thymic lobule, they are more numerous in the medulla than in the cortex. Eosinophils are present in appreciable number within the thymus throughout the last four days of the incubation period, but the number within the thymus at any one time is variable. Most of the eosinophils are of the polymorphonuclear type.

Other cell types appear in the thymus during the closing hours of incubation. Many erythroblasts are present at 426 hours. Occasionally, multinucleated giant cells are found in the cortex after 450 hours. It is believed that these cells play a phagocytic role in the late stages of the incubation period.

In many of the embryos after 400 hours, cystic spaces are present in some of the thymic lobules just above the anterior pole of the thyroid. The spaces are lined with a simple squamous to cuboidal epithelium, and they vary in size and number in the different thymi. All these spaces lie within the thymic lobule. When these thymi are traced through serial sections, the cystic spaces usually terminate blindly, but occasionally they end as solid epithelial cell nests which appear to be continuous with the reticulum. These cystlike spaces may arise from the failure of the original thymic tubule to lose its lumen or from the rapidity with which the thymus grows.

SUMMARY

The morphogenesis of the thymus of 420 developing chicken embryos was studied by means of serial, sagittal, transverse, and frontal sections, taken at approximately twelve-hour intervals from ninety-six hours of incubation until hatching.

After 120 hours of incubation, the double origin of the thymus can be observed. The large portion, or thymus III, is derived from the ventral and lateral wall of the third visceral pouch. Thymus IV, a smaller portion than thymus III, is derived from the dorsal wall of the intermediate part of the fourth visceral pouch and eventually fuses with thymus III.

An occasional large lymphocyte migrates into the periphery of the epithelial thymic tissue from the surrounding mesenchyma at 177 hours. Small thymic cells appear at 252 hours.

Connective tissue ingrowth from the primitive capsule and growth and increase in the number of the epithelial cells produce the primary lobules at 213 hours of incubation.

Lymphocytes migrate into the thymic tissue along the interlobular septa.

Indirect evidence is presented to support the hypothesis that small lymphocytes (thymic cells) arise by cellular division from large lymphocytes.

A cortical and medullary zone can be distinguished in the thymus at 319 hours, but these zones are not definitely separated until 357 hours.

The medulla develops from the epithelial syncytium of the thymic primordium.

Hassall's corpuscles develop from the medullary epithelial cells.

Eosinophil cells do not appear in the thymus until late embryonic life. This also is true of other cell types commonly found in thymic tissue.

No evidence was obtained to indicate that thymic development is dependent on the function of other endocrine glands.

Based upon the morphogenetic findings, it appears improbable that a complete surgical ablation of the thymus of the chicken could be accomplished without simultaneous removal of the thyroid and parathyroid glands. This may account for the inconclusive results on thymectomy in the chicken.

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A Comparison of Newcastle Disease Virus Recovery from Bone Marrow and from Pools of Respiratory Tract and Spleen

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DURING THE STUDY of outbreaks of Newcastle disease (ND) in Turkey by one of us (H.B.) in 1944-1948, it was desirable to obtain specimen material from outlying districts for virus isolation. Since the distances are great and transportation is slow (5 days to one month travel time, depending on the season) and since solid CO_2 is not available for preservation in these areas, viscera were almost valueless as a source of virus. It was recognized that Newcastle disease virus (NDV) is pantropic, and therefore it was believed that it should be present in the bone marrow. Since bone marrow usually does not become contaminated by decomposition and since chicken bones are easy to transport, these were tried. Virus was recovered from several such cases. Most of these outbreaks were of the fulminating, Asiatic type of disease, but today the milder form predominates.

While one of us (H.B.) was visiting at the New Jersey Agricultural Experiment Station Poultry Pathology Laboratory, the question naturally arose: Is the virus of the milder form of the disease, which is epornitic in the United States, uniformly present in the bone marrow?

EXPERIMENTAL

Forty cases were studied in an effort to answer this question. In 4 cases, only bone material was examined for virus, but in all others, tracheae or tracheal mucus and spleens were examined for virus in our routine manner. This consists of grinding pooled tracheae and spleen samples in a mortor with sterile sand and about 5 cc. of broth. Mucus and spleen samples are emulsified with a pipette in about 2 cc. of broth. After centrifugalization, 1 cc. of the supernatant is mixed with 10 mg. of streptomycin and 10,000 units of penicillin and inoculated into the allantoic sac of 9- to 11-day-old embryos in a dose of 0.2 cc. per egg. From the first 9 cases, only one femur per case was examined, but in all the others, a femur was saved from each bird in a case from which other tissues were saved. The bones and other tissues were separately frozen and stored from the time of collection until inoculation. At the time of inoculation, the bones were split with cartilage shears and a bit of marrow scooped out with a small spatula. Marrow from each bone in a case was pooled in a tube and emulsified with a pipette in about 2 to 4 ml. of nutrient broth. When very young chick bones were used, the volume was reduced to 1 ml. After centrifugation, 1 ml. of the supernatant was removed to another tube and mixed with 10,000 units of penicillin and 10 mg. of streptomycin, each separately contained in 0.1 ml. of physiological saline. The antibiotic-treated inoculum was then used in a dose of 0.2 cc. per embryo to inoculate each of 4, 10-day-old chicken embryos.

RESULTS

The detailed results are summarized in table 1. Of the 40 cases in which bone marrow was examined, no other examination for virus was made in 4 cases. Three of these were diagnosed at the time of autopsy as ND and one as infectious bronchitis. Bone marrow examinations gave 1 positive for a case of birds vaccinated one week previously by the stick method. In 16 cases, tracheae and spleens were examined for NDV; 8 cases yielded virus and 8 did not. Of the 8 cases where virus was not recovered, 1 was diagnosed as ND at postmortem, 1 was questionable, and 6 were considered not to be ND. Virus was recovered from bone marrow in 3 cases. Tracheal mucus and spleens were examined for NDV from 20 other cases; 14 were positive, and 6 were negative. Newcastle disease was diagnosed at postmortem in 3 of the 4 negative cases and 1 case was questionable. Bone

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TABLE I—Bone Marrow Versus Spleen and Respiratory Tissue for Newcastle Disease Virus Recovery

			History				Autopsy		Virus recovery				
Case No.	Date (1951)	Age (weeks)	% Mortality*	Nervous symptoms	Respiratory symptoms**	Clouded air sacs	Tracheal exudate	Clouded cornea	Bone marrow	Trachea and spleen	Mucus and spleen	No. birds examined	Final diagnosis
1	5/25	6.0	1.2	.....	+	+	+	.....	—	.....	+	4	+
2	5/25	10.0	1.9	+	.....	+	.....	.....	—	None attempted		.....	+
3	5/25	11.5	0.0	+	+	+	.....	.....	—	+	.....	2	+
4	5/25	14.5	6.5	—	—	+	.....	+	—	.....	—	3	+
5	5/25	12.5	7.2	.....	.....	+	.....	+	—	.....	+	2	+
6	5/28	5.0	....	Vaccinated 1 wk. previously			.....	.....	+	None attempted		.....	+
7	5/28	6.0	....	—	.....	+	+	.....	—	.....	+	5	+
8	5/28	2.0	10.0	+	+	+	+	.....	+	+	.....	6	+
9	5/28	5.0	4.3	+	.....	+	.....	.....	—	+	.....	4	+
10	5/29	3.5	3.0	—	+	.....	+	.....	—	None attempted		.....	—
11	5/29	H†	....	.....	+	+	.....	.....	—	.....	+	2	+
12	5/29	P‡	3.0	.....	.....	+	.....	.....	—	.....	+	3	+
13	5/29	5.0	0.0	—	—	+	.....	.....	+	+	.....	4	+
14	5/29	12.0	20.0	+	+	+	.....	.....	—	.....	+	5	+
15	5/29	12.0	1.1	+	.....	+	.....	.....	—	None attempted		.....	+
16	5/29	5.0	....	+	+	+	.....	.....	—	+	.....	4	+
17	5/29	14.0	....	.....	+	.....	+	.....	—	.....	—§	2	—
18	5/29	17.0	6.4	+	.....	+	.....	.....	—	.....	—	2	+
19	5/31	2.5	16.6	+	+	+	.....	+	—	+	.....	3	+
20	6/4	6.0	....	+	.....	+	.....	.....	—	.....	+	4	+
21	6/4	4.0	0.0	.....	+	.....	.....	.....	—	—	.....	2	—
22	6/4	7.0	0.0	—	+	.....	.....	.....	—	—	.....	1	—
23	6/5	3.0	1.5	—	+	+	+	.....	—	—	.....	5	—
24	6/5	17.0	1.0	+	.....	+	.....	.....	—	.....	—	2	+
25	6/8	20.0	14.0	+	.....	+	.....	.....	—	.....	+	4	+
26	6/18	26.0	3.0	+	.....	+	.....	.....	—	.....	+	4	+
27	6/21	15.0	5.3	+	+	+	.....	.....	—	.....	+	5	+
28	6/25	12.0	0.8	+	.....	+	.....	.....	—	—	.....	4	+
29	6/26	3.0	4.0	.....	.....	.....	.....	.....	—	—	.....	4	—
30	6/26	11.0	3.0	—	.....	+	.....	.....	—	—	.....	1	—
31	6/28	7.0	....	+	.....	+	.....	.....	—	.....	+	5	+
32	7/2	7.0	75.0	.....	.....	+	.....	.....	+	.....	+	5	+
33	7/2	9.5	5.0	+	.....	+	.....	.....	+	.....	+	4	+
34	7/5	8.0	1.2	.....	.....	+	.....	.....	+	+	.....	5	+
35	7/10	16.0	9.0	+	.....	+	.....	.....	—	+	.....	3	+
36	7/13	7.0	30.0	+	.....	+	.....	.....	—	.....	+	5	+
37	7/17	24.0	3.5	+	+	+	.....	.....	—	.....	—	4	?
38	7/17	11.0	....	+	.....	+	.....	.....	—	.....	—	1	—
39	7/24	15.0	10.0	.....	.....	+	.....	.....	—	—	.....	3	—
40	7/27	P	4.3	+	.....	.....	.....	.....	—	—	.....	4	?
									6+ 34—	8+ 8—	14+ 6—	128	29+ 9— 2?

*%Mortality—that reported at the time of laboratory visit, usually soon after the disease was recognized; NOT the ultimate, which was generally much higher than the figures given. . . . in the mortality or nervous symptoms columns indicates that no information was available. **Respiratory symptoms may not always have been recorded. †Hens; production dropped from 1,400 to 500 eggs. ‡Pullets; production dropped from 203 to 60 eggs. §Mucus only examined.

marrow examinations were positive for virus in 2 cases. Obviously, virus was always recovered from respiratory and spleen tissue when it was recovered from bone marrow.

It would appear from these results that NDV is not so consistently present in bone marrow as in respiratory and spleen tissue. Further, it would seem that bone marrow is unsuitable for recovery of virus in so far as the currently epornitic strains of NDV in this area are concerned. This suggests also that the milder form of ND now present in Turkey may have to be dealt with in a somewhat different manner from that hitherto used, when virus recoveries are to be attempted on specimens shipped from great distances.

Immunological and Pathological Findings on a Highly Virulent Strain of Newcastle Disease Virus from Mexico

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OFFICIAL INFORMATION from the Mexican Ministry of Agriculture late in 1950 advised that there had been occurring in Mexican poultry an epornitic which was causing very heavy losses even in mature flocks, and that it had been identified as Newcastle disease. Early in 1951, two strains of virus were brought from Mexico to the laboratories of the Bureau of Animal Industry (BAI) in Washington, D. C., where comparative immunological and pathological studies have been conducted.

The purpose of this paper is to report our findings and to point out the difficulties that would be encountered in making a positive diagnosis of a virulent outbreak of Newcastle disease from the introduction of virulent foreign strains of the virus as differentiated from the disease ordinarily occurring in this country.

MATERIALS

Viruses.—Through the Joint Commission for the Eradication of Foot-and-Mouth Disease in Mexico, arrangements were made for a United States member of the staff to bring, under refrigeration, two strains of Newcastle disease virus (NDV) from Mexico to Washington, D. C. The virus cultures were in two small rubber-stoppered vials and consisted of several cubic centimeters of sterile chicken embryo allantoamnionic fluids which bore these labels:

No. 1. Cepa de virus de Newcastle, Queretaro, Mexico, Aisl. 15/XII/50. Pase 27/I/51
Septicemic form

No. 2. Cepa Newcastle, Ixtapalapa, Mexico. Pase No. 17. 13/I/51
Respiratory form only

(Hereafter these are referred to, respectively, as the Queretaro and Ixtapalapa NDV's.)

The United States NDV used for comparative study was the California strain 11914,[1] 25th chicken embryo passage.

Chickens.—All chickens used in this study were the progeny of a New Hampshire flock maintained as Newcastle disease-susceptible stock at the Animal Disease Station, Beltsville, Md., specifically as a source of birds for disease research.

Eggs.—The fertile eggs used in this investigation were from the flocks of the Animal Husbandry Division of the BAI. These flocks were immunized when 16 weeks old with live virus Newcastle disease vaccine (wing-web administered).

Antiserum.—BAI lot 7 Newcastle disease antiserum was prepared in September, 1949, from serial bleeding by cardiac puncture of mature roosters which had recovered from exposure by intramuscular injection of the California 11914 NDV.

TESTS FOR IDENTITY OF THE VIRUSES

Immediately upon receipt of the viruses from Mexico, tests were started in 12-day-old chicken embryos to determine: (1) the embryo minimum lethal dose (e.m.l.d.) of the material received, and (2) the number of e.m.l.d.'s of these viruses which could be neutralized with standard BAI Newcastle disease antiserum, as compared with the number normally neutralized with this antiserum against the California 11914 NDV. In both viruses, more than 1,000,000 e.m.l.d.'s were neutralized by the antiserum, the same as is regularly obtained against the California 11914 NDV. This positively identified both of the cultures as NDV. Although representing tests conducted at a later date, portions of table 8 replicate the data obtained on these preliminary tests.

While the neutralization tests in embryos were in progress, hemagglutination and hemagglutination-inhibition tests were conducted on the cultures as received from Mexico. The results of these tests concurred with those of the aforementioned tests in identifying the cultures as NDV. Again, while table 9 actually portrays the results of tests conducted at a later date, portions of the data do duplicate the results obtained on these preliminary tests.

The aforementioned tests were conducted according to the standard procedures which have been published by the BAI.[2, 3]

TESTS FOR PATHOGENICITY

Experiment 1.—Upon completion of the identification tests, 50, 4-week-old chickens, 20, 14-week-old broilers, and 20 hens were placed in a special isolation unit and inoculated intra-

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muscularly with 0.1 cc. of a 1 : 10,000 dilution (56,000 e.m.l.d.'s) of the Queretaro NDV. On the same date in a separate unit, 40, 6-week-old chickens were inoculated intramuscularly with 0.1 cc. of a 1 : 1,000 dilution (316,000 e.m.l.d.'s) of the Ixtapalapa NDV. Observations were made for thirteen days, at which time the test was terminated so that the rooms could be disinfected and prepared for other tests. The results of the experiment are presented comparatively in table 1. A cursory review of this table immediately reveals that the Queretaro NDV was extremely virulent, while the Ixtapalapa NDV by comparison was only moderately pathogenic.

It was concluded that the Queretaro NDV was much more virulent than any strain of NDV which has been observed in this country, except the Asiatic strain of NDV which was introduced into California in 1950 and immediately eradicated.[4, 5] The Ixtapalapa NDV appeared to be very similar to the California 11914 NDV. Except for cross-neutralization tests in embryos with serums from the birds surviving exposure to the Ixtapalapa NDV, which are discussed under Immunological Tests, additional studies were not made on this virus.

Experiment 2.—In an effort to determine whether the Queretaro NDV would consistently manifest its virulent characteristics, or whether it might revert to a less severe type, two methods of serial passages were made in mature chickens, namely, artificial passage and passage by natural means (contact). To start the first method of passage, 2 intramuscularly inoculated roosters were killed three days after inoculation. Previous experience with Newcastle disease had indicated that the highest concentration of virus in the tissues of chickens is reached about the third or fourth day following exposure to the disease. Portions of their spleens, livers, and lungs were removed and pooled. These pooled tissues, in about three volumes of nutrient broth, were ground in a mechanically rotated TenBroeck glass tissue grinder. (The lungs were minced with a scissors before attempting to grind them in the TenBroeck grinders.) The resulting suspension was centrifuged lightly and 0.1 cc. of the unfiltered supernatant material was injected intramuscularly into each of another series of 5 susceptible roosters. In turn, 2 of these birds were killed on the third or fourth* day following inoculation and the procedure repeated until seven passages were completed. Starting with the second passage, a portion of testis was included in the tissues pooled. The 3 remaining roosters of each passage were held until they died from the disease. For the seventh passage in order to add numerical significance to the results, 20 hens were used. The course of the disease for each passage is shown in table 2. Neither the incubation period nor the course of the disease varied significantly during the series of passages.

To determine the titer of the inoculums used in the passages, tenfold dilutions in nutrient broth containing approximately 10 mg. per cubic centimeter of streptomycin were made of the supernatant material used for inoculums for each passage. Each dilution was then injected in 0.1-cc. amounts into the allantoic sac of 12-day-old chicken embryos, 5 embryos per dilution, to determine the quantity of NDV present in each tissue pool. The results of these titrations are shown in table 3. Probably the variation in the quantity of virus[6] present in the different passages represents the expected normal variation in different birds. Also, the concentration of virus appears to be greater on the fourth day than the third.

Experiment 3.—To start the second series of passages, where contact with infected birds

*These steps were generally carried out on Mondays and Thursdays. Hence, the interval in days for one time was three and the other four.

TABLE 1—Results of Comparative Pathogenicity Tests* of the Queretaro and Ixtapalapa Newcastle Disease Viruses for Chickens

Days post-inoculation	Observations			
	Queretaro NDV groups			Ixtapalapa NDV
	50 chickens 4-weeks-old	20 chickens 14-weeks-old	20 hens	40 chickens 6-weeks-old
2	a few off feed	.....	.....	.....
3	several acutely ill	.....	.....	.....
4	3 deaths	.....	1 death	audible rales from group
5	29 deaths	majority acutely ill	.....	2 paralyzed
6	17 deaths	6 deaths	8 deaths	3 deaths
7	(only 1 alive)	1 death	5 deaths	5 deaths
8	.....	3 deaths	.....	1 death
9	.....	1 death	1 death	1 death
10	.....	.....	.....	5 deaths
11	.....	.....	.....	.....
12	1 death	7 paralyzed	1 paralyzed	1 death
13	.....	2 apparently recovering	4 apparently recovering	10 crippled 14 normal
Mortality	100%	Probably 90%	Probably 75%	40% (crippling 25%)

*The respective groups of chickens were exposed by intramuscular injection with 56,000 e. m. l. d.'s of the Queretaro NDV or 316,000 e. m. l. d.'s of the Ixtapalapa NDV.

was to be the means of transmission, 5 susceptible mature roosters were placed in a cage with 3 roosters which were inoculated intramuscularly three days previously with the Queretaro NDV. These 5 roosters, after being exposed for three or four days,** were placed in a clean cage with 5 susceptible roosters. After the latter were exposed for three or four days, they were transferred to another clean pen and another lot of 5 susceptible roosters placed with them. This procedure was followed until six passages were completed The sixth passage was made with 20 susceptible hens in order to give numerical significance to the results. Table 4 shows the course of the disease in successive order for each passage. Comparison with the data in table 2 shows that the incubation period, course of the disease, and percentage mortality for the contact passage series are almost identical with those of the artificial passage series. The average survival time for the 32 birds in the artificial passage series which died with the acute disease following exposure by hypodermic inoculation was 7.5 days, while, for the 41 dying with the acute disease from contact exposure, it was 8.3 days. The fact that the incubation period was nearly the same length for the two methods of exposure rather pointedly illustrates that the birds in the contact passage series must have become infected almost immediately after being placed

See footnote.

TABLE 2—Course of Disease Produced by the Queretaro Newcastle Disease Virus During Seven Serial Passages Through Mature Chickens by Artificial Inoculation. The Passages Were Made by Intramuscular Inoculation of 0.1 cc. of a Suspension of Ground Organs from Birds of the Previous Passage

Bird No.	Passage	No. days postinoculation required for symptoms and death 3 4 5 6 7 8 9 10 11 12 13 14	Remarks
R1493	First	*———D**	
R1517	First	—D	
R1523	First	———D	
R1508	First	K†	
R1510	First	K	
R1527	Second	———D	
R1525	Second	———D	
R1449	Second	———D	
R1509	Second	K	
R1518	Second	K	
R1479	Third	———D	
R1496	Third	————————	Crippled, K
R1478	Third	————————	Crippled, K
R1482	Third	K	
R1489	Third	K	
R1490	Fourth	———D	
R1502	Fourth	————————	Crippled, K
R1486	Fourth	————D	
R1505	Fourth	K	
R1440	Fourth	K	
R1090	Fifth	———D	
R1094	Fifth	———D	
R1089	Fifth	——D	
R1167	Fifth	K	
R1113	Fifth	K	
R1025	Sixth	——D	
R1069	Sixth	———D	
R1068	Sixth	———D	
R----	Sixth	K	
R1092	Sixth	K	
H1109	Seventh	——D	
H1063	Seventh	——D	
H1163	Seventh	——D	
H----	Seventh	———D	
H1051	Seventh	———D	
H1258	Seventh	——D	
H1269	Seventh	——D	
H1133	Seventh	——D	
H1119	Seventh	——D	
H 983	Seventh	————————	Paralyzed, K
H1158	Seventh	———D	
H1237	Seventh	——D	
H1101	Seventh	———D	
H1131	Seventh	————————	Paralyzed, K
H7197	Seventh	——D	
H 999	Seventh	————————	Standing, K
H1116	Seventh	———D	
H 998	Seventh	——D	
H3737	Seventh	——D	
H4035	Seventh	———D	

* ———— period of visible illness; **D = death; †K = killed.

with the birds in the incubative stage of the disease.

On three occasions during this experiment, susceptible mature chickens, numbering 8, 2, and 5, respectively, were placed in the room in cages several feet from those containing the infected birds. In about a week, each of these birds developed clinical manifestations of the disease and all but 2 died. This serves to emphasize the extreme infectiousness of the disease.

Experiment 4.—It was considered desirable to know the susceptibility of pigeons to the Queretaro NDV. Individual blood samples, approximately 2½ cc. in volume, were collected by intracardial puncture with a small-gauge hypodermic needle from a group of young pigeons. The serum from each of these samples was tested by serum-neutralization test in chicken embryos to insure that each pigeon was susceptible to Newcastle disease. The pigeons had been procured through a pigeon broker, and the possibility existed that the birds might have had previous exposure to Newcastle disease. None of the serums of the 24 pigeons used in this study neutralized more than 10 e.m.l.d.'s of the California 11914 NDV, while serums from blood samples taken at the end of this experiment from the surviving pigeons all neutralized 10,000 e.m.l.d.'s of the same NDV. These results, therefore, certainly indicate that all the pigeons were susceptible at the beginning of the experiment. After a few days were allowed for the pigeons to recover from the blood sampling, they were divided into two groups of 16 and 8, respectively, and placed in separate isolation rooms. Eight pigeons of the first group received intramuscularly approximately 10,000,000 e.m.l.d.'s of the Queretaro NDV; the remaining 8 pigeons of this group were left with the inoculated birds for exposure by contact. The results of this experiment are shown in table 5. Six of the 8 inoculated pigeons died with the acute disease, and all but 1 manifested paralysis for one or two days prior to death. Three of the 8 birds exposed by contact died, thus supplying evidence that transmission of the Queretaro NDV by contact will occur in pigeons.

To compare the virulence of the Queretaro NDV with the California 11914 NDV, 5 pigeons of the second group were inoculated intramuscularly with the same quantity of the latter NDV on the same day as the first group. The remaining 3 pigeons were left in the cage with the 5 inoculated birds for exposure by contact. Three of the inoculated pigeons died on the fifth, eighth, and twelfth days, respectively, following inoculation. The other 2 inoculated pigeons and 3 pigeons that were exposed by contact remained normal throughout an eighteen-day observation period. Here again, the pigeons that succumbed all manifested paralysis for a day or so before death.

Comparison of the results of these tests, both natural and artificial routes of exposure being considered, indicates that the Queretaro NDV is somewhat more virulent for pigeons than the California 11914 NDV. As a whole, however, they are similar to those reported by Beach[7] and other workers (as cited by

TABLE 3.—Titrations of the Quantity of Newcastle Disease Virus in the Pooled Tissues of the Birds Killed for Artificial Passage Series of the Queretaro Newcastle Disease Virus

Bird passage	Organs titrated	No. days post-inoculation when tissues were taken	Dilution of inoculums 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	e.m.l.d.'s NDV/cc.
First	liver spleen lung	3	5/5*	5/5	5/5	5/5	2/5	0/5	1/5	0/5	676,000
Second	liver spleen lung	4	5/5	5/5	5/5	5/5	5/5	3/5	0/5	0/5	14,800,000
Second	testis	4	5/5	5/5	5/5	5/5	5/5	3/5	0/5	0/5	14,800,000
Third	liver spleen lung testis	4	5/5	5/5	5/5	5/5	5/5	4/5	1/5	1/5	31,600,000
Fourth	liver spleen lung testis	3	5/5	5/5	5/5	4/5	0/5	0/5	0/5	0/5	237,000
Fifth	liver spleen lung testis	3	5/5	5/5	5/5	3/5	2/5	0/5	0/5	0/5	316,000
Sixth	liver spleen lung testis	4	5/5	5/5	5/5	5/5	2/5	0/5	0/5	0/5	676,000

*Deaths / embryos inoculated.

Beaudette[8]) with regard to the susceptibility of pigeons for NDV.

IMMUNOLOGICAL TESTS

To initiate steps for controlling an extensive outbreak of virulent Newcastle disease, such as the Queretaro NDV would be capable of producing, it would be of unlimited value to know whether the Newcastle disease vaccines which are commercially available in this country could be expected to protect poultry against such a virulent virus. To obtain this information, each of three groups of chickens, ages 22 days, 10 weeks, and 1 year, containing, respectively, 40, 38, and 40 birds, were equally divided between two isolation rooms and vaccinated with commercial live virus Newcastle disease vaccine (wing-web administered). (Titration in chicken embryos showed that this particular lot of vaccine contained approximately 30,000 e.m.l.d.'s of NDV/cc.)

It was convenient to include in the experiment another group of mature birds which were each given intramuscularly 1 cc. of a BAI experimental crystal-violet Newcastle disease vaccine prepared according to the method of Doyle and Wright.[9] At the time of this experiment, the vaccine had been prepared for nine months. This group of birds was held at the Animal Disease Station, Beltsville, Md. Vaccination of all groups was carried out on the same date.

On the day of challenge, twenty-one days following administration of the vaccines, the birds receiving the crystal-violet vaccine were brought to Washington, D. C., equally divided into two groups, and placed in the isolation rooms with the birds that had been given the live virus vaccine. Susceptible birds of each

TABLE 4—Course of Disease Produced in Mature Chickens by the Queretaro Newcastle Disease Virus During Six Serial Passages Where Contact with Previously Exposed Chickens Was the Means of Exposure

Bird No.	Passage	No. days postexposure required for symptoms and death (3 4 5 6 7 8 9 10 11 12 13 14)	Remarks
R1524	First	D**	
R1526	First	•———D	
R1520	First	— D	
R1521	First	—D	
R1515	First	D	
R1507	Second	————————————Died 20th day postexposure	
R——	Second	—D	
R1481	Second	———D	
R1491	Second	————————D	
R1484	Second	—D	
R1245	Third	———D	
R1483	Third	———D	
R1480	Third	———D	
R1472	Third	–D	
R1246	Third	———D	
R1109	Fourth	— D	
R1185	Fourth	——D	
R1175	Fourth	— D	
R1129	Fourth	— D	
R1157	Fourth	D	
R1056	Fifth	————D	
R1044	Fifth	—D	
R1304	Fifth	—D	
R1188	Fifth	——— D	
R1041	Fifth	———D	
H1213	Sixth	———D	
H1113	Sixth	— D	
H1134	Sixth	—— D	
H1136	Sixth	———D	
H 942	Sixth	———D	
H——	Sixth	— D	
H——	Sixth	———D	
H 976	Sixth	———D	
H 968	Sixth	———D	
H 997	Sixth	—D	
H1030	Sixth	—D	
H 972	Sixth	———D	
H 806	Sixth	———D	
H1212	Sixth	———D	
H1082	Sixth	—D	
H1073	Sixth	————	Paralyzed, destroyed
H1259	Sixth	———D	
H3761	Sixth	————	Paralyzed, destroyed
H4894	Sixth	————	Standing, destroyed
H1088	Sixth	———D	

•———— period of visible illness; **D = death.

TABLE 5—Result of Experiment to Determine the Susceptibility of Pigeons to the Queretaro Newcastle Disease Virus

Band No.	Method of exposure	No. days postinoculation required for symptoms and death										
		3	4	5	6	7	8	9	10	11	12	13	14
586	10^7 e.m.l.d.'s NDV intramuscularly					•—	—D						
564	10^7 e.m.l.d.'s NDV intramuscularly										N†		
418	10^7 e.m.l.d.'s NDV intramuscularly						—D						
300	10^7 e.m.l.d.'s NDV intramuscularly												N
250	10^7 e.m.l.d.'s NDV intramuscularly	D											
919	10^8 e.m.l.d.'s NDV intramuscularly			—D									
360	10^7 e.m.l.d.'s NDV intramuscularly			—D									
118	10^7 e.m.l.d.'s NDV intramuscularly				——	——	—D						
356	Contact with above birds												N
979	Contact with above birds											——	—D
170	Contact with above birds												N
500	Contact with above birds												N
887	Contact with above birds					D							
246	Contact with above birds												N
683	Contact with above birds					D							
142	Contact with above birds												N

•—————— period of visible illness; **D = death; †N = Normal.

TABLE 6—Result of Immunological Test Conducted with Groups of Chickens Immunized with Live Virus Newcastle Disease Vaccine (Wing-Web Administered) and a Group Immunized with a BAI Experimental Crystal-Violet Newcastle Disease Vaccine. Birds Challenged Three Weeks Following Vaccination with Queretaro Newcastle Disease Virus

Dates	Groups receiving commercial live virus Newcastle disease vaccine			BAI exptl. crystal-violet vaccine, lot 1	Groups used as susceptible controls		
	20 chickens, 22-days-old	18 chickens, 10-weeks-old	20 hens	20 hens	10 chickens, 6-weeks-old	11 chickens, 13-weeks-old	10 hens
3-8 to 3-18	Respective groups vaccinated 1 dead from vaccine virus						
3-29	All groups challenged intramuscularly with approximately 10,000 e.m.l.d.'s Queretaro NDV.						
4-3				.....	1 dead	1 dead	.....
4-4				2 dead	.....	.....	6 dead
4-5				1 dead	4 dead	3 dead	2 dead
4-6				.....	3 dead	6 dead	.....
4-7				.....	1 dead	1 dead	1 dead
4-8				.....	.....	.....	.....
4-9				.....	1 dead	.....	.....
4-10				4 crippled	.....	.....	1 dead
Affected by challenge (%)	0	0	0	35	100	100	100

TABLE 7—Result of Immunological Test Conducted for Comparative Purposes. Chickens Immunized with Commercial Live Virus or Experimental Crystal-Violet Newcastle Disease Vaccines and Then Challenged with California 11914 Newcastle Disease Virus. (Compare with table 6.)

Dates	Groups receiving commercial live virus Newcastle disease vaccine			BAI exptl. crystal-violet vaccine, lot 1	Groups used as susceptible controls		
	20 chickens, 22-days-old	20 chickens, 10-weeks-old	20 hens	20 hens	9 chickens, 6-weeks-old	10 chickens, 13-weeks-old	11 hens
3-8	Respective groups vaccinated						
3-29	All groups challenged with approximately 10,000 e.m.l.d.'s California 11914 NDV.						
4-3		.....	.....	.....	4 dead	1 dead	.....
4-4		1 dead	.....	1 dead	.....	.....	2 dead
4-5		.....	.....	.....	.....	.....	.....
4-6		.....	.....	.....	1 dead	.....	.....
4-7		.....	.....	1 dead	.....	.....	.....
4-8		.....	.....	.....	.....	.....	.....
4-9		.....	.....	.....	2 dead	.....	.....
4-10		.....	1 crippled	2 crippled	1 crippled	1 crippled	3 dead
Affected by challenge (%)	0	5	5	20	80	20	50

age group were also introduced into each isolation room to serve as controls for the challenge viruses.

The birds in one isolation room were challenged intramuscularly with approximately 10,000 e.m.l.d.'s of the Queretaro NDV, and those in the other isolation room were challenged with the same quantity of the California 11914 NDV by the same route.

The results of these tests are shown comparatively in tables 6 and 7. Briefly summarized, the results were that the birds which were immunized with live virus vaccine were solidly immune to intramuscular challenge with the Queretaro NDV, whereas all the susceptible controls died with the acute disease from this virus. The crystal-violet vaccine gave only partial protection against this virus. Two of the birds that received live virus vaccine in the group that was challenged with the California 11914 NDV apparently failed to be immunized, as they were affected by challenge. Here again, the crystal-violet vaccine afforded only partial protection against the California 11914 NDV. This experiment clearly indicates that commercial live virus Newcastle disease vaccine (wing-web administered) would provide a method of immunization against the virulent type of Newcastle disease caused by the Queretaro NDV. The experiment also indicates that a crystal-violet vaccine prepared according to the method cited could be expected to provide 65 per cent protection against this virulent virus.

Attention is directed to the illustration of comparative virulence of the California 11914 NDV and the Queretaro NDV that is provided in tables 6 and 7 by noting the way these viruses affected the susceptible control groups.

Cross-neutralization tests with serum taken from the surviving birds of the first test for pathogenicity were performed in chicken embryos to give additional support to the immunological identity of the two Mexican NDV's. It was found that serum from the few birds which survived infection with the Queretaro NDV neutralized approximately the same titrated quantity of the California 11914 NDV as that of its homologous virus or the Ixtapalapa NDV. The same was true of the serums collected from birds which survived exposure to the Ixtapalapa NDV. Included in these tests, both as a control and for comparative purposes, were standard BAI Newcastle disease antiserum and BAI normal chicken serum. The results of these tests are shown comparatively in table 8.

The same comparisons were made by cross-hemagglutination-inhibition tests with the aforementioned antiserums from the two Mexican NDV's. The results are given in table 9.

Although it is evident in both the cross-neutralization tests and the cross-hemagglutination-inhibition tests that there are slight differences in the activity of the antiserums from the two Mexican NDV's, there appears to be no doubt as to their immunological identity, and it is highly questionable whether any significance should be given these slight variations.

TABLE 8—Results of Cross-Neutralization Tests Conducted with Serums from Birds Surviving Exposure to the Queretaro or the Ixtapalapa Strains of Newcastle Disease Virus Against Their Respective Viruses and the California 11914 Newcastle Disease Virus. BAI Standard Antiserum and Normal Serum Are Included for Comparison and as Controls in the Tests

Test components	Virus dilution											e.m.l.d.'s neut.
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	l.d.$_{50}$	
California 11914 NDV	...	...	...	...	...	3/3*	3/3	2/3	0/3	0/3	$10^{-9.25}$	.....
Newcastle disease antiserum BAI lot 7	2/3	1/3	0/3	0/3	...	...	...	...	...	...	$10^{-1.5}$	5,600,000
Normal chicken serum BAI lot 6	...	...	...	...	...	3/3	3/3	1/3	0/3	0/3	$10^{-7.75}$	.....
Serum birds surviving Queretaro NDV	3/3	2/3	1/3	0/3	0/3	0/3	...	...	...	...	$10^{-2.5}$	560,000
Serum birds surviving Ixtapalapa NDV	3/3	2/3	1/3	0/3	0/3	0/3	...	...	...	...	$10^{-2.5}$	560,000
Queretaro NDV	...	...	...	...	3/3	3/3	3/3	3/3	1/3	0/3	$10^{-8.75}$	.....
Newcastle disease antiserum BAI lot 7	3/3	2/3	0/3	1/3	0/3	0/3	...	...	...	...	$10^{-2.25}$	2,000,000
Normal chicken serum BAI lot 6	...	...	...	...	3/3	3/3	3/3	1/3	1/3	0/3	10^{-8}	.....
Serum birds surviving Queretaro NDV	2/3	2/3	1/3	0/3	0/3	0/3	...	...	...	...	$10^{-2.25}$	3,160,000
Serum birds surviving Ixtapalapa NDV	3/3	3/3	2/3	1/3	1/3	0/3	...	...	...	...	$10^{-2.75}$	100,000
Ixtapalapa NDV	...	...	...	...	3/3	3/3	3/3	3/3	0/3	1/3	$10^{-8.5}$	.....
Newcastle disease antiserum BAI lot 7	2/3	1/3	1/3	1/3	0/3	0/3	...	...	...	...	10^{-2}	3,160,000
Normal chicken serum BAI lot 6	...	...	...	...	3/3	3/3	3/3	3/3	0/3	1/3	$10^{-8.5}$	.....
Serum birds surviving Queretaro NDV	3/3	2/3	1/3	0/3	0/3	0/3	...	...	...	...	$10^{-2.5}$	1,000,000
Serum birds surviving Ixtapalapa NDV	3/3	3/3	3/3	1/3	0/3	0/3	...	...	...	...	$10^{-3.75}$	56,000

*Deaths / embryos inoculated.

GROSS PATHOLOGY AND HISTOLOGICAL EXAMINATIONS

The spleen, intestine, proventriculus, portions of pectoral muscle, and the eyes from birds dying from the Queretaro NDV were taken for histological examination. Grossly, these tissues manifested lesions which were prominently discernible to the eye. Briefly, they were as follows:

Spleen.—Multiple pinpoint gray areas were present on the capsule and the organ appeared shrunken rather than enlarged.

Intestine.—There were large, plaquelike subserous hemorrhages with clots projecting into the lumen of the intestine. A rather uniform location for one of these was the portion of intestine which passes between the cephalad parts of the cecums. The same lesion was also observed in the cecums. The intestinal lesions were most prominent in the younger birds.

Proventriculus.—The mucosal surface regularly showed small areas of hyperemia which, in many cases, appeared to have progressed to hemorrhages. These lesions tended to extend posteriorly into the sublining tissue of the gizzard.

Pectoral Muscle.—Various degrees of degeneration of the breast muscles were observed in nearly all the birds autopsied. The muscle had a pale, parboiled appearance and, particularly in the laying hens, was extremely friable. Occasional areas of necrosis were observed. This is a lesion that no one seems to have described for Newcastle disease.

Eye.—In the older birds, about 50 per cent manifested opaque corneas and, in several cases, both eyes were involved. Three birds showing this lesion were killed in a moribund state and the paired eyes immediately removed and preserved in Zenker-formol fixative. Opacity of the eyes as a lesion of Newcastle disease has not been described.

Histologically, the lesions in these different tissues were as follows:

Spleen.—Focal necrosis with hyaline (or fibrinoid) deposits occurred throughout the organ.

Intestine.—The subserous hemorrhages were accompanied by necrotizing changes. The foci appeared to start as hemorrhage in the subepithelial tissues, followed by necrosis and sloughing of the mucosa. Then, the formation of a large fibrinonecrotic clot, which extended into the lumen of the gut, took place.

Proventriculus.—Organs which grossly appeared to contain mucosal hemorrhages were selected for sections. Microscopically, hemorrhages were not observed but rather the lesions consisted of extreme hyperemia of the mucosal vessels.

Pectoral Muscle.—The lesion was that of Zenker's degeneration.

Eye.—The opacity of the eyes was caused by severe edema of the cornea. Compared with sections which were available of the normal chicken eye, it was estimated that the corneas were about three times their normal thickness. Other changes were also

TABLE 9—Results of Comparative Hemagglutination Tests with the Queretaro, Ixtapalapa, and California 11914 Newcastle Disease Viruses. Also Hemagglutination-Inhibition Tests with the Homologous Antiserums of the Respective Viruses Against Each of the Viruses

Test components	Virus dilution										
	Undiluted	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Control
California 11914 NDV	+	+	+	+	+	+	+	+*	—	—	—
California + BAI lot 7 antiserum 1:10	—	—	—	—	—	—	—	—	—	—	—
California + BAI lot 6 normal serum 1:5	+	+	+	+	+	+	+	+	—	—	—
California + Queretaro antiserum 1:10	—	—	—	—	—	—	—	—	—	—	—
California + Ixtapalapa antiserum 1:10	—	—	—	—	—	—	—	—	—	—	—
Queretaro NDV	+	+	+	+	+	+	+	+	+	—	..
Queretaro + BAI lot 7 antiserum 1:10	+	—	—	—	—	—	—	—	—	—	..
Queretaro + BAI lot 6 normal serum 1:5	+	+	+	+	+	+	+	+	±	—	..
Queretaro + Queretaro antiserum 1:10	—	—	—	—	—	—	—	—	—	—	..
Queretaro + Ixtapalapa antiserum 1:10	—	—	—	—	—	—	—	—	—	—	..
Ixtapalapa NDV	+	+	+	+	+	+	+	+	+	±	..
Ixtapalapa + BAI lot 7 antiserum 1:10	—	—	—	—	—	—	—	—	—	—	..
Ixtapalapa + BAI lot 6 normal serum 1:5	+	+	+	+	+	+	+	+	+	—	..
Ixtapalapa + Queretaro antiserum 1:10	—	—	—	—	—	—	—	—	—	—	..
Ixtapalapa + Ixtapalapa antiserum 1:10	—	—	—	—	—	—	—	—	—	—	..

+ = hemagglutination; — = no hemagglutination; *Final reading made at sixty minutes. Test conducted at room temperature.

noted in the eye. The aqueous humor contained an abnormal amount of albuminoid substance with scattered erythrocytes in it. The choroid, while normally a very vascular structure, appeared to be extremely hyperemic and, in 1 case, it contained hemorrhages. The iris in all cases showed cellular infiltration. This latter lesion is the only eye pathology which has been described by other workers.

Except for the changes in the eye and the degeneration of the breast muscle, the pathology was similar to that described by Jungherr *et al.*[10] for the Hertfordshire strain of NDV which was studied at the Huntington Laboratories. Their report contains excellent illustrations of the lesions observed in the proventriculus and intestines.

It must be emphatically brought out, however, that these lesions are not observed in connection with our American strains of NDV.

CLINICAL SYMPTOMS

Chickens.—In the great majority of cases produced by the Queretaro NDV, the birds simply sickened without manifesting any nervous symptoms, became prostrate, and died in forty-eight to seventy-two hours —as is ordinarily observed for acute septicemic diseases. The nervous symptoms that are associated with Newcastle disease, *i.e.*, torticollis and paralysis of the legs and wings, were manifested only in the minority of the birds and those were the less acute or chronic cases. Cyanosis of the comb was a rather constant observation in the acute cases. Extreme edema of the wattles and face was seen in 2 roosters used in the contact-transmission studies. This is the lesion that has been described as characterizing fowl plague. In the case of these 2 roosters, it is possible that the severity of the swelling was related to injury from fighting. Even so, it was observed near the terminus of the disease. Respiratory râles audibly evident during the acute stages of the disease were common. It is suspected that these were due to congestive edema of the lungs rather than an inflammatory condition of the respiratory tract.

The simultaniety with which this disease occurred in all birds of each group of the contact exposure series is a characteristic that probably should be weighed heavily in tentatively suggesting that virulent Newcastle disease be suspected should an outbreak of this nature be observed in the field.

Pigeons.—Paralysis was the most prominent symptom in the pigeons infected with the Queretaro NDV, although 3 of the birds died with the acute disease without showing any symptoms. Several of the birds assumed the position of lying on their backs and even when placed upright would immediately flop over on their backs.

DISCUSSION

The economic expansion of the poultry industry in recent years, the rank that its products have attained among agricultural products, the relatively recent intensification of phases of poultry production in certain areas, and the present extensive use of international air transportation are factors that greatly increase the vulnerability of this country to an outbreak of an exotic poultry disease. It must not be overlooked that the poultry industry is a potential area for attack by biological warfare. Also, it is to be borne in mind that the promptness with which a disease outbreak can be diagnosed is paramount to initiating control measures against it.

The study herewith reported has been made with consideration of all these factors. It pointedly bears out the fact that, even though we have several very reliable laboratory tests available for diagnosing Newcastle disease, they will not differentiate the extremely pathogenic strains of NDV from the less pathogenic ones. As the data show, the only means discovered for differentiating the virulent Queretaro NDV from milder strains of NDV were pathogenicity tests in significant numbers of susceptible fowl, together with the postmortem lesions that have been described in this paper. The virulence of the Queretaro NDV is again emphasized by pointing out that, of a total of 128 mature susceptible birds used in these studies, 113, or 87 per cent, died with the acute disease from this virus.

The extensive use of live virus Newcastle disease vaccine (wing-web administered) by poultrymen during the past three years suggests that a significant percentage of our poultry population probably is immune to Newcastle disease. The fact that chickens of different ages, immunized with this type of Newcastle disease vaccine, survived

challenge by inoculation of the Queretaro NDV without manifesting any evidence of illness would indicate that our immune flocks provide a barrier which would help considerably in controlling an outbreak of disease from a virus of the nature of the Queretaro NDV should such be detected in this country.

SUMMARY

1) The Queretaro Newcastle disease virus (NDV) is much more virulent than any strain of NDV which has been observed in this country, except the Asiatic strain of the virus which was introduced into California in 1950 and immediately eradicated.

2) The only means discovered for differentiating the Queretaro NDV from the milder forms of the disease were pathogenicity tests in significant numbers of susceptible fowl.

3) Birds of different ages, immunized with commercial Newcastle disease vaccine (live virus, wing-web administered), survived challenge by inoculation of the virulent Mexican virus, without manifesting any evidence of illness. An experimental crystal-violet Newcastle disease vaccine afforded 65 per cent protection against this same virulent virus.

4) On the basis of the tests conducted, the Ixtapalapa NDV appears very similar to the California strain 11914 NDV.

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Electron Microscope Studies of Four Strains of Infectious Bronchitis Virus

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ELECTRON MICROSCOPE studies of the virus of infectious bronchitis of chickens previously reported by Reagan *et al.*[1] showed the virus particles when suspended in distilled water to be round in form, with an occasional small filamentous projection, and a mean diameter of 70 to 90 mμ. The present studies were undertaken to determine if other strains of infectious bronchitis virus (IBV) differ morphologically under electron microscope examination.

MATERIALS AND METHODS

The bronchitis strains, obtained from Dr. F. R. Beaudette of the New Jersey Agricultural Experiment Station and Dr. C. H. Cunningham of Michigan State College, had been proved by them to be bronchitis. They were stored in the —40 C. dry ice cabinet until the start of the experiment.

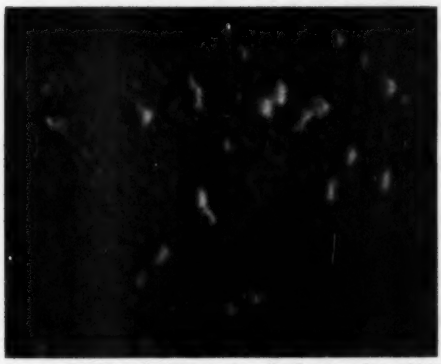

Fig. 1—B1580 and 1587 strains of infectious bronchitis shadowed with chromium at tangent 1/6. x 42,000.

One strain, the seventy-third egg passage of the original IBV isolated by Dr. Beaudette, was designated as B 1580 and 1587. The other strain supplied by Dr. Beaudette was the twentieth passage of the Wachtel IBV which was labeled BR 18 and BR 19. Each of these strains was subjected to two additional egg passages at this laboratory. The remaining strains were supplied by Dr. Cunningham and each had been passed twice in embryonating chicken eggs. One, the Van Roekel strain, designated VR Lot 277-1,[2] was passed eight additional times in eggs. The other, a field strain labeled 50-51-173,[2] was passed four more times in eggs. Nine- to 11-day embryonating White Leghorn eggs were used. In all cases, the inoculum consisted of 0.1 cc. and the route was via the allantoic cavity.

Embryos of eggs injected with B 1580 and 1587 from Dr. Beaudette and VR Lot 277-1[2] from Dr. Cunningham were dead in forty-eight hours, whereas embryos of eggs injected with BR 18 and BR 19 from Dr. Beaudette and

Fig. 2—Wachtel strain of infectious bronchitis shadowed with chromium at tangent 1/6. x 50,000.

50-51-173[2] from Dr. Cunningham were not dead until sixty hours had elapsed. On the death of the embryos, allantoic fluid was collected from ten eggs of each strain and was filtered through a size L3 Seitz filter with a Republic Filter, Inc., asbestos pad designated ST. The filtrates were centrifuged for two hours at 44,620 revolutions per minute (r.p.m.) in a Spinco ultracentrifuge. There was less than a 1.5 C. change in temperature of the refrigerated outer jacket. Each sediment was resuspended in distilled water, allowed to stand for ten to fifteen minutes, and placed on parlodion screens which had been prepared three days previously. The screens were then shadowed with chromium, as described by Williams and Wyckoff,[3] at arc tangent 1/6 and examined under an RCA EMU electron microscope. Various fields were chosen for electron micrographs.

Figure 1 (B 1580 and 1587) and fig. 2 (VR 277-1[2]) show virus particles which tend

From the Maryland State Board of Agriculture, Live Stock Sanitary Service, University of Maryland, College Park.

toward being oblong, with short prolongations and with a dense appearance. Figure 2 (BR 18 and BR 19) shows particles which tend toward being round, without filamentous projectings, with irregular borders and a less dense appearance than the other strains. Figure 4 (50-51-173[3]) shows virus particles similar to those seen in fig. 2. The virus particles of the four strains were of approximately the same size, ranging in diameter from 60 to 100 mμ.

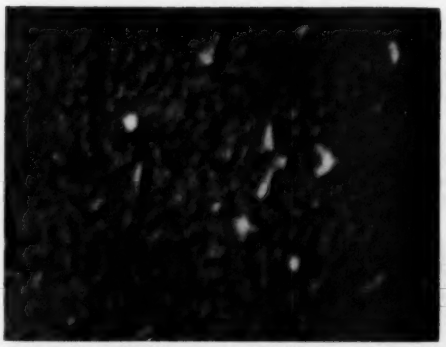

Fig. 3—Van Roekel strain of infectious bronchitis shadowed with chromium at tangent 1/6. x 45,000.

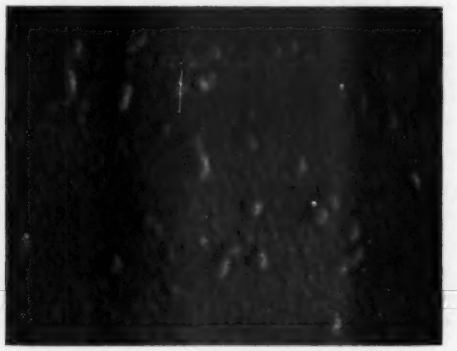

Fig. 4—Field strain of infectious bronchitis shadowed with chromium at tangent 1/6. x 45,000.

DISCUSSION

Comparison of the morphology of virus particles shown in a previous study with those in the current examinations is not satisfactory because, in the first instance, electron micrographs were prepared from unshadowed virus. The differences noted in the strains currently examined may be, in some way, associated with the difference in pathogenicity for embryos. The short period between inoculation of the eggs and death of the embryos infected with two of the strains can not be explained entirely by increasing pathogenicity with successive embryo passages. The VR Lot 277-1[2] strain, even in the third embryo passage, caused death of the embryos within forty-eight hours, whereas the BR 18 and BR 19 strain, after 22 passages, did not produce death until sixty hours or longer. Long filamentous projections or tails, such as are seen in Newcastle disease virus were not evident.[4] As an additional differential factor, bronchitis virus particles are smaller than Newcastle disease particles.

SUMMARY

Electron micrographs of four infectious bronchitis strains are presented. There is some difference in the strains in regard to shape and density of the virus particles, but not in regard to size. Two strains show short prolongations.

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The Response of the Rabbit Uterus to Instillation of Semen at Different Phases of the Estrous Cycle

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THE PRESENT STUDY was initiated to determine the effect of the stage of the estrous cycle on the uterine response, both grossly and histologically, to the intrauterine instillation of bacterially contaminated and bacteria-free semen. Previous work by Black *et al.*[1] showed that marked pyometra resulted when semen collected in an artificial vagina was introduced into the uterine horn of a pseudopregnant rabbit, but no gross reaction occurred when such semen was instilled into the uterine horn of an estrous rabbit. This observation appeared to be in accordance with the theory that ovarian hormones influence the ability of the uterus to overcome infections.

In order to determine whether the reaction which occurred in the pseudopregnant rabbit was due to the bacteria in the semen, the experiment was designed to include the injection of both bacterially sterile epididymal semen and ejaculated semen into the uterine horns of both estrous and pseudopregnant rabbits. The study was further indicated because of various present-day practices of invading the uterus in bovine artificial insemination procedures and of irrigation of the bovine uterus with antibiotics.

REVIEW OF THE LITERATURE

Reports of studies of a similar nature could not be found in the literature, although Fluhmann[2] reported the effect of ovarian hormones on the response of the rabbit uterus to traumatic stimulation. The passage of a silk ligature through one horn of the uterus in "normal" and in spayed rabbits resulted in a local increase of macrophages at the site of injury. Similar injury to one uterine horn in a stage of progestational proliferation resulted in increased numbers of macrophages at the site of injury and also throughout both horns. He interpreted this to mean that the rabbit uterus when under the influence of ovarian hormones, which cause intense stimulation of tissue growth, acquires the power to respond to traumatic injury more actively.

Further work by Fluhmann[3] showed that estrogen stimulation alone, as well as progesterone alone, gave an increased macrophage response at the site of injury and also increased the number of macrophages in the uninjured horn.

Marshall[4] described the gross changes accompanying estrous in the rabbit which consisted of swelling and congestion. A "wholesale destruction" of the uterine mucosa was described to occur at the end of two weeks of pseudopregnancy. The leukocytic infiltration response and the presence of leukocytes in the lumen of the glands are illustrated in proestrus.

MATERIALS EMPLOYED AND THE DEVELOPMENT OF METHODS

Female domestic rabbits of mixed breeding, which ranged from 6 months to 2 years of age, were used. Some were virgin while others were multiparous. These animals appeared healthy, and were housed and cared for under average conditions. The females were separated in individual cages at least one month before beginning the experiment in order to eliminate accidental induction of pseudopregnancy.

Experimental pseudopregnancy was induced by mating females with a vasectomized male. Such females were allowed to remain in pseudopregnancy for ten days, at which time uterine instillation was performed. For comparison with the pseudopregnant rabbits, estrous rabbits were mated with a vasectomized male on the day of insemination and, thereafter, handled in the same manner as the pseudopregnant ones. General anesthesia was induced by in-

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travenous injection of 10 ml. of an aqueous solution of 0.7 per cent sodium pentobarbitol, 1.4 per cent magnesium sulfate, and 2.8 per cent chloral hydrate. Supportive ether anesthesia was used if necessary.

A midline abdominal incision was made, and the left uterine horn exposed. The trial material was injected into the lumen of the anterior end of the left horn through a syringe and a 20-gauge needle by puncturing the uterine wall. The uterus was replaced and the incision closed with nylon sutures and skin clips.

In all experiments with rabbits, the right horn served as a control for the left horn. The rabbit is an especially useful animal in this regard, because there is no direct connection between the lumens of the two horns. Likewise, the uteri of the estrous rabbits were compared with those of the pseudopregnant rabbits. Thus, a double control on the response was possible; the right horn served as a control for the left (experimental) horn in both estrous and pseudopregnant animals, and the uteri of the estrous rabbits constituted controls on the reaction of the pseudopregnant individuals. The first 6 rabbits were used to determine the ideal postinsemination time at which they should be destroyed to provide uterine specimens. Three pseudopregnant and 3 estrous rabbits were artificially inseminated through a laparotomy site with semen collected in an artificial vagina.

One estrous and 1 pseudopregnant rabbit were killed at twenty-four hours postinsemination, another pair at forty-eight hours, and the third pair at seventy-two hours.

At the time of killing, the macroscopic nature of any reaction which had occurred was recorded and sections were taken from the middle of each horn. This portion was selected to avoid as much as possible the effect of needle trauma at the anterior end of the experimental horn. The tissue blocks were fixed in mercuric formalin and the histological sections stained with hematoxylin and eosin. The same technique was followed with all the experimental rabbits.

Histological examination revealed that the inflammatory reaction had reached its peak within twenty-four hours, and repair had begun by the forty-eighth and seventy-second hours. Consequently, it was decided to destroy the experimental rabbits at twenty-four hours after insemination.

The rabbit semen was collected in an artificial vagina and, naturally, it was not free of bacteria. When such semen was streaked on blood-agar, a mixed bacterial growth was obtained. The quantity of semen employed was 0.25 ml. of a dilution of one to four with a modified Kreb's solution. The apparent impossibility of securing bacterially sterile ejaculated semen prompted the use of bacteria-free epididymal spermatozoa for comparative purposes. A male rabbit was destroyed and the testes, epididymides, and vasa deferentia were removed immediately with forceps closing the ampullae. One-fourth milliliter of sterile distilled water was then injected slowly into the ampulla. This fluid was forced by massage along the vas deferens and into the epididymis. Alternate injection and withdrawal yielded a concentration of spermatozoa approaching that of the one to four dilution of ejaculated semen. This epididymal "semen" did not contain the secretions of the accessory sexual glands and, when streaked on blood-agar plates, bacterial growth failed to develop.

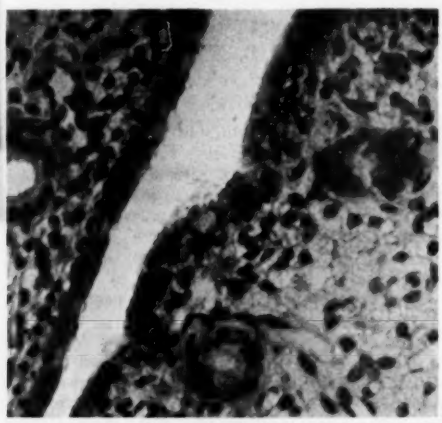

Fig. 1—Section of the left uterine horn of an estrous rabbit twenty-four hours following injection of semen contaminated with bacteria. A moderate number of leukocytes are in the stroma and the epithelium is intact. Hematoxylin and eosin stain. x 390.

RESULTS

Three estrous and 4 pseudopregnant rabbits were used to study the effect of semen contaminated with bacteria. Twenty-four hours after insemination, the inseminated uterine horns of the estrous rabbits were devoid of any gross reaction (table 1). Histologically, only scattered polymorphonuclear leukocytes were present in the uterine lumen, and the epithelium was intact with with only a moderate number of polymorphonuclear leukocytes and a few mononuclear leukocytes in the endometrial stroma. Moderate hyperemia and edema were apparent (fig. 1). The lumens of the untreated horns of the same rabbits were free of cellular debris. Histologically, the epithelium was intact and there were a few mononuclear leukocytes, with an occasional polymorphonuclear leukocyte in the endo-

metrial stroma. This part of the organ showed moderate edema and hyperemia (fig. 2).

The lack of reactivity of the estrous rabbits was in extreme contrast to that of the 4 pseudopregnant rabbits which received a similar inoculation of bacterially contaminated semen in the left horn. Twenty-four hours later, the inseminated horns had developed a gross pyometra. The horns were filled with a cellular exudate consisting mainly of polymorphonuclear leukocytes. Histological study revealed that the glands were enlarged and filled with cellular exudate. The epithelium had disintegrated in some areas; in others it was undergoing degeneration. The endometrium and myometrium contained many polymorphonuclear leukocytes, with a few mononuclear leukocytes within the endometrium. Hyperemia was present throughout (figs. 3 and 4). The right, or control horn, was not unusual grossly but, microscopically, it revealed some mucous secretion in the lumen but no cellular infiltration (fig. 5). Thus, the reactions of the uteri of both the estrous and the pseudopregnant rabbits were of the same character but differed greatly in magnitude, that of the pseudopregnant being much greater. Under the influence of estrogen, the uterus appears to be capable of ridding itself, with ease, of irritants including bacteria; while, under the influence of progesterone, the uterus has great difficulty in responding in such a manner.

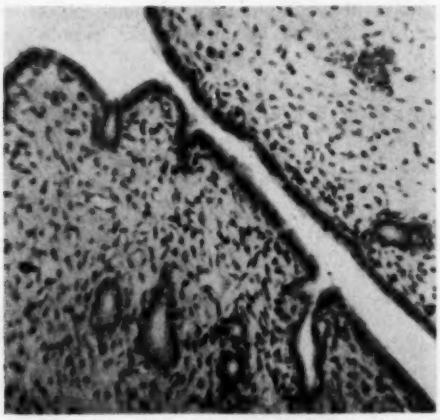

Fig. 2—Section of the right or control uterine horn of an estrous rabbit. A few leukocytes are in the stroma. Hematoxylin and eosin stain. x 390.

To permit comparison of the effect of contaminated semen and bacteria-free semen, the next group of rabbits consisting of 3 estrous and 4 pseudopregnant animals were injected in the left horn with sterile epididymal spermatozoa. The horns of the estrous rabbits which received sterile semen were grossly normal one day later. Microscopically, the lumen was free of exudate, the epithelium intact, and many polymorphonuclear leukocytes and a few mononuclear leukocytes were present in the endometrium. Moderate hyperemia and edema were observed (fig. 6). The control horns of the estrous rabbits were normal to gross examination and, microscopically, a few

TABLE I—Gross and Histological Findings Twenty-Four Hours Postinjection of Estrous or Pseudopregnant Rabbits' Uteri with Sterile Epididymal or Contaminated Ejaculated Semen

	Histological findings				
	Cellular debris in lumen	Loss of uterine epithelium	Leukocytic infiltration of endometrium	Edema and hyperemia	Gross appearance
Ejaculated semen (contaminated)					
1. Estrous rabbits					
a. Injected horn	+	—	++	++	hyperemia*
b. Control horn	—	—	+	++	hyperemia*
2. Pseudopregnant rabbits					
a. Injected horn	++++	++	+++	++	hyperemia pyometra
b. Control horn	—	—	—	—	normal
Epididymal semen (sterile)					
1. Estrous rabbits					
a. Injected horn	—	—	++	++	hyperemia*
b. Control horn	—	—	+	++	hyperemia*
2. Pseudopregnant rabbits					
a. Injected horn	—	—	+	—	normal
b. Control horn	—	—	—	—	normal

— denotes the absence of a condition.
+ denotes the presence of a condition, and the number indicates the degree.
* hyperemia considered normal for estrus.

leukocytes were seen in the endometrium and myometrium. Moderate edema and hyperemia were present. These findings indicate that edema, hyperemia, and some leukocytic infiltration were physiological processes accompanying estrus.

Fig. 3—Section of the left uterine horn of a pseudopregnant rabbit twenty-four hours following injection of semen contaminated with bacteria. Much cellular debris is in the lumen, there is epithelial degeneration, and many leukocytes have infiltrated the stroma. Hematoxylin and eosin stain. x 90.

Three of 4 pseudopregnant rabbits' horns receiving sterile semen were grossly normal and had a slight amount of mucus in the lumen. Histologically, the epithelium was intact and free of infiltrating polymorphonuclear leukocytes. A very few mononuclear leukocytes were present in the endometrium. Hyperemia or edema were absent (fig. 7). The fourth animal had a few polymorphonuclear leukocytes in the lumen, many polymorphonuclear leukocytes in the endometrium, and hyperemia throughout. Gross or histological abnormalities were not demonstrated in the control horns of the same rabbits. The reaction of the 1 animal was very similar to that observed in the estrous animal which had received semen that contained bacteria. Of course, it is possible that a few bacteria might have accidentally contaminated the inoculum employed. The epididymal semen failed to incite an inflammatory response either in the estrous or pseudopregnant rabbit, ex-

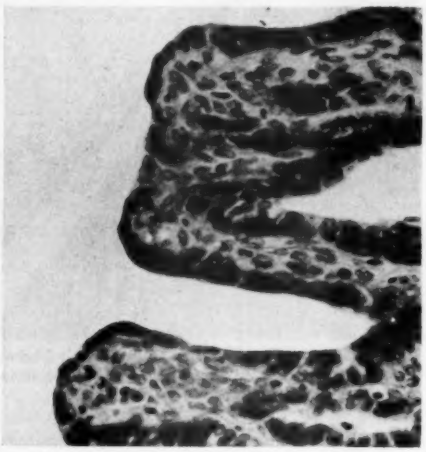

Fig. 4—Section of the left uterine horn of a pseudopregnant rabbit twenty-four hours following injection of semen contaminated with bacteria. Some epithelium is denuded; other epithelium is undergoing degeneration. Leukocytes are present in the stroma. Hemotoxylin and eosin. x 390.

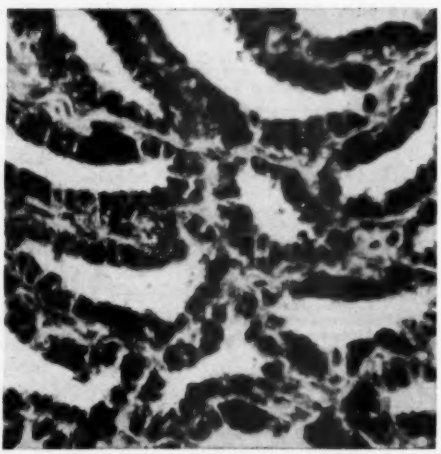

Fig. 5—Section of the right or control uterine horn of a pseudopregnant rabbit. Leukocytic infiltration is absent. Hematoxylin and eosin stain. x 390.

cept in the one instance. This is in striking contrast to the response to the semen, contaminated with bacteria, which was collected in the artificial vagina.

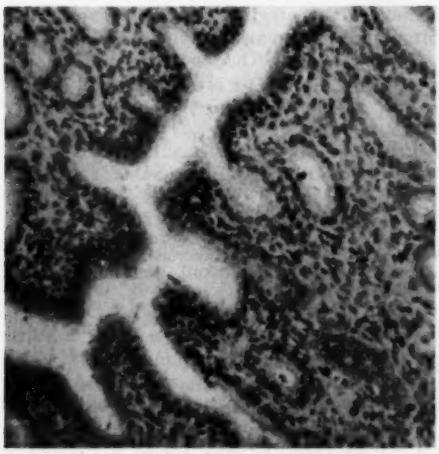

Fig. 6—Section of the left uterine horn of an estrous rabbit twenty-four hours following injection of sterile epididymal spermatozoa. A moderate number of leukocytes are in the stroma, the epithelium is intact, and leukocytes are absent from the lumen. Hematoxylin and eosin stain. x 390.

DISCUSSION

The data indicate that the uterus of the pseudopregnant rabbit reacts with a massive influx of leukocytes to the introduction of semen which is contaminated with bacteria incident to its collection with an artificial vagina. Such a uterus is under the influence of high levels of ovarian hormones, progesterone being the most influential. The reaction is so severe that epithelial denudation occurs, the lumen is filled with a cellular exudate, and gross pyometra is observed. The control right horns of these uteri fail to show any reaction. Preliminary work indicates that recovery could occur within a few days. The reaction does not spread to the other horn, as Fluhmann reported when a silk ligature was used as an irritant to one horn.

The injected horn of the estrous rabbit, which is under the influence of high levels of estrogen, responds with a slight additional infiltration of leukocytes other than that which accompanies normal estrus. There is a small quantity of cellular exudation in the lumen of the inseminated horn, while the lumen of the control horn is devoid of reaction.

Sterile epididymal spermatozoa was the source of sterile semen for this work, and the data show that injection of sterile semen into the uterus of an estrous rabbit superimposes very little reaction upon the physiological edema, hyperemia, and leukocytic infiltration of estrus. Likewise, the pseudopregnant rabbit's injected horn responds only mildly with leukocytic infiltration when sterile semen is injected. This suggests that the original acute inflammatory process which followed injection of bacterially contaminated semen into the uterus of the pseudopregnant rabbit was due to the bacteria or their products. The sterile semen used obviously lacked the accessory secretions of the ejaculated semen.

Evidence also indicates that the uterus under the influence of high levels of progresterone (as in pseudopregnancy) responds to bacterially contaminated semen with a greater leukocytic mobilization, but that the uterus is less able to protect itself than is the uterus of estrus, which is under the influence of high levels of estrogen. Perhaps a share of the defensive mechanism of

Fig. 7—Section of the left uterine horn of a pseudopregnant rabbit twenty-four hours following injection of sterile epididymal spermatozoa. The epithelium is intact, and there are very few infiltrating leukocytes. Hematoxylin and eosin stain. x 390.

the "estrous" uterus is due to its secretions and the relaxation of the cervix which allows free drainage.

Our findings raise the question of whether pyometra may not sometimes result from a hormonal imbalance which predisposes the uterus to infection. If such is the case, invading bacteria would not be able to establish themselves if the hormone balance were normal. Added evidence of the influence of estrogens in disease processes is also offered. Our own unpublished data as well as those of Simon[5] indicate that the uterus of the cow reacts much as does the uterus of the rabbit in response to bacteria.

SUMMARY

The uteri of 14 rabbits were studied and observations were made of the gross and microscopic changes that followed injection of bacterially contaminated semen or of sterile epididymal sperm into the uteri of estrous rabbits and of ten-day pseudopregnant rabbits.

The injection of sterile epididymal spermatozoa in the uteri of estrous or pseudopregnant rabbits failed to induce a significant reaction.

The injection of bacterially contaminated semen in the uteri of pseudopregnant rabbits induced a marked pyometra.

The injection of bacterially contaminated semen in the uteri of estrous rabbits caused only a mild inflammatory reaction.

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Infectious Myxomatosis I. Vaccination II. Antibiotic Therapy

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INFECTIOUS MYXOMATOSIS is a virus disease of the domestic rabbit which is frequently characterized by the formation of tumors on the skin of the ears, nose, and feet. The disease is highly host-specific and is readily transmissible from one individual to another of the same species. In contrast to tumor diseases in general of domestic animals, infectious myxomatosis is invariably fatal.

While the original strain of myxoma virus, isolated in Uruguay in 1898 by Sanarelli,[1] has been studied extensively in the United States, England, and Australia, control of the disease itself, either by therapeutic or preventice measures has, by comparison, received little attention. This may be attributed largely to the fact that in England the naturally occurring disease does not exist, whereas in Australia it has only recently been introduced in an effort to control the wild rabbit population. In the United States, the disease appears to be confined to California, where it has been indigenous since its introduction from Mexico in 1931.[2] Unsuccessful attempts at immunization were made shortly thereafter by Fisk and Kessel,[3] using inactivated blood and tissues from infected rabbits. Hyde,[4] however, obtained some degree of resistance to the South American strain of the virus by the use of a tissue vaccine. While Shope[5] succeeded in protecting a high percentage of rabbits against this virus by the inoculation of living fibroma virus intradermally, his method, nevertheless, was impractical for routine use.

Failure to develop a satisfactory vaccine prior to 1940 was not, however, of great significance, inasmuch as commercial rabbit raising was conducted on a relatively small scale; consequently infectious myxomatosis did not constitute a particularly pressing problem. During the past ten years, however, the industry has developed to the point where today it is one of major economic importance, particularly in California. As a result, the infectious diseases of the domestic rabbit now assume considerably greater significance than heretofore. This is particularly true of infectious myxomatosis, a disease which, on attaining epizoötic proportions, decimates the domestic rabbit population as does no other disease of this species known to occur in the state.

A particularly devastating epizoötic, which occurred in 1949 in California, brought into sharp focus the helpless plight of the industry when confronted with a widespread outbreak. Since the recommended procedures such as mosquito abatement, slaughter of infected stock, and the imposition of rigid sanitary precautions are designed primarily to serve as containing measures, these actually have little effect once the disease attains epizoötic proportions. In the interests of the industry, it therefore became imperative that steps be taken to explore the possibility of developing specific measures of a practical nature to combat this infection. Accordingly, an investigation was undertaken with this as the object.

Inasmuch as previous attempts at specific control lacked modern knowledge concerning immunization against virus diseases and the benefits of antibiotics, the problem was attacked through these two relatively unexplored approaches.

MATERIALS

A strain of myxoma virus recovered in July, 1949, from one of the many field outbreaks in the vicinity of San Diego, Calif., was the one used throughout this study. Heart blood drawn from several animals at the peak of the febrile period was pooled and used to reproduce the disease experimentally in laboratory rabbits by intradermal inoculation. Following several serial passages in this species, growth of the virus was adapted to the chorioallantois of 9- to 11-day-old embryonating hens' eggs. In adapting the virus to this host, the original inoculum consisted of virulent rabbit blood drawn at the height of the febrile period; thereafter a 1¢ to 20 per cent suspension of chorioallantoic membrances in either saline buffered at pH 7.2 or allantoic fluid was used. The inoculated eggs were incubated at 37 C. for four

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The technical assistance of Joseph K. Saito, Davis, is gratefully acknowledged.

days and the membranes then harvested. After 16 blind transfers, during which time the only indications of the presence of virus were thickening of the inoculated membranes and their infectivity for rabbits, visible foci appeared on the membranes of the seventeenth passage and were regularly produced thereafter. The infection did not, however, kill the embryo.

In titrating infectious material, a 1 per cent concentration thereof in buffered saline was centrifuged lightly for five minutes to sediment coarse particles. Serial tenfold dilutions were then prepared in the same diluent from the supernatant. One-half cubic centimeter of each dilution was inoculated intradermally into the shaved backs of mature rabbits, using a minimum number of 2 animals for each dilution. The minimum lethal dose (m.l.d.) was calculated on the basis of the number of rabbits which died with typical symptoms of the disease within the two-week period immediately following inoculation.

Aureomycin,* penicillin, chloromycetin,** and terramycin† were the antibiotics utilized in this study. The parenteral form only of each was employed, which consisted, in the case of the aureomycin and terramycin, of the crystalline hydrochloride with glycine buffer. The chloromycetin was provided in solution in propylene glycol, while the penicillin used was the crystalline procaine G product in peanut oil containing two per cent aluminum monostearate.

Except where otherwise stated, adult New Zealand White rabbits were used throughout the study.

IMMUNIZATION STUDIES—EXPERIMENTAL

Three lots of infected tissues consisting of chorioallantoic membrances from the twenty-first serial egg passage, the thirty-first to thirty-sixth inclusive and the forty-first passage, labeled 1, 2, and 3, respectively, were utilized in the preparation of vaccines. The membranes of the first two lots were held as harvested in sealed vials under dry ice refrigeration until required. Those of the third lot were minced in a Waring blendor, and a 10 per cent suspension by weight thereof was prepared in their allantoic fluid prior to storage under dry ice refrigeration.

On the basis of titration in rabbits, lots 1 and 2 were found to contain 200,000 m.l.d. of virus per gram of tissue, while the virus content of the third was ten times this concentration per cubic centimeter of suspension.

Various methods were used to inactivate the virus in the preparation of the vaccines described later. On the basis of the claim that young rabbits are quite resistant to infectious myxomatosis, an attempt was made to produce immunity in a litter of nursing rabbits by the use of living virus.

Each vaccine was tested in relatively few rabbits, thus permitting the selection of the most promising preparations by the use of the minimum number of animals.

VACCINES

Formolized Virus (Preparation 1).—Pooled membrances from lot 1 were ground in a glass tissue grinder and brought to a concentration of 10 per cent by weight with 0.2 per cent formolized saline buffered at pH 7.2. The suspension was then held at room temperature with constant shaking for ninety-six hours.

Formolized Virus (Preparation 2).—A second preparation was made by the addition of formalin to the lot 3 membrane suspension to a final concentration of 0.2 per cent.

Heat-Inactivated Virus.—A portion of the 10 per cent suspension of membrances from lot 3 was subjected to a temperature of 60 C. by immersing in a water bath for thirty minutes, followed by rapid cooling in iced water.

Mustard-Inactivated Virus.—To aliquots of a 10 per cent suspension of lot 2 membrances in saline buffered at pH 7.2, a nitrogen mustard (methyl bis (B—chloroethyl) amine hydrochloride) in the form of a 2 per cent solution in distilled water was added to a final concentration of 100, 500, 1,000, 1,500 and 2,000 mg. of mustard per liter of suspension. Each aliquot was held at 37 C. for twelve hours. The preparations were than tested for viability and for the local effect on the tissues by inoculating 0.5 cc. intradermally into the shaved backs of rabbits. While all concentrations of mustard used resulted in inactivation of the virus when tested as described previously, severe to moderate necrosis followed the inoculation of all but the preparation containing the lowest content of mustard. Therefore, in the preparation of a vaccine, a concentration of 250 mg. of mustard per liter of suspension was used.

Crystal Violet-Inactivated Virus.—One part of a 0.25 per cent solution of crystal violet in undiluted glycerin° was added to four parts of a 10 per cent suspension of lot 3 membrances. The mixture was incubated at 37 C. for twenty-one days with occasional shaking, and samples removed therefrom were checked periodically during this time for viability. At the end of this period, the virus was found to be no longer infective for rabbits.

Live Virus.—A suspension of membrances of lot 3 which had been held under dry ice refrigeration was diluted with buffered saline to contain 1,000 m.l.d. of virus per cubic centimeter, and inoculated immediately.

With the exception of the live virus, all vaccines were stored at 4 C. and held for no longer than three weeks before using.

Vaccination and Challenge.—The vaccines

*Supplied by Lederle Laboratories. **Supplied by Parke, Davis and Company. †Supplied by Chas. Pfizer and Company.

were administered by various routes, either in single or multiple injections, in the dosage and at the time intervals shown in table 1. All animals were challenged, with one exception, from two to four weeks following the last injection of vaccine by the intradermal inoculation of 50 or 100 m.l.d. of virus contained in a volume of 0.5 cc. of a saline suspension of infected chorioallantoic membranes. In the case of 1 animal receiving the heat-inactivated vaccine, 100 m.l.d. of virus was given twenty-four hours following vaccination to determine if interference on the part of the heat-inactivated virus would prevent infection by the virulent challenge virus.

RESULTS

All rabbits, irrespective of the vaccines used or the routes by which they were inoculated, succumbed when challenged with either 50 or 100 m.l.d. of virus. The details are presented in table 1.

EXPERIMENTAL

Aureomycin, penicillin, chloromycetin, and terramycin were tested against the myxoma virus in rabbits in which the disease had been produced experimentally by the intradermal inoculation of either 100 or 1,000 m.l.d. of virus. Relatively few animals were used in the tests on each antibiotic in which it was attempted to ascertain the most promising preparation by a process of screening. Treatment was routinely started on the sixth or seventh day following infection, by which time the animals invariably manifested marked clinical symptoms of the disease. Dosage and the route of inoculation

TABLE 1—Response of Vaccinated and Control Rabbits to Inoculation with Active Myxoma Virus

Vaccine	No. rabbits used	Total amount vaccine inoculated per rabbit	Inoculation schedule	Route of inoculation	Amount of challenge virus inoculated	Survival ratio
Formolized virus (preparation 1)	3	0.5 cc.	Single injection	Intradermal	100 m.l.d.	0/3
	2 (controls)	.....	.....	Intradermal	100 m.l.d.	0/2
Formolized virus (preparation 2)	1	1.0 cc.	Single injection	Subcutaneous	50 m.l.d.	0/1
	1	2.0 cc.	Two injections of 1.0 cc. each, spaced 1 week apart	Subcutaneous	50 m.l.d.	0/1
	2 (controls)	.....	.....	Intradermal	50 m.l.d.	0/2
Formolized virus (preparation 2)	6	4.0 cc.	Four injections of 1.0 cc. each, at weekly intervals	Intravenous	100 m.l.d.	0/6
	2 (controls)	.....	.....	Intradermal	100 m.l.d	0/2
Heat-inactivated virus	1	1.0 cc.	Single injection	Intravenous	100 m.l.d.*	0/1
	1	2.0 cc.	Two injections of 1.0 cc. each, spaced 1 week apart	Intravenous	100 m.l.d.*	0/1
	1	2.0 cc.	Two injections of 1.0 cc. each, spaced 1 week apart	Subcutaneous	100 m.l.d.*	0/1
	2 (controls)	.....	.....	Intradermal	100 m.l.d.	0/2
Mustard-inactivated virus	3	1.0 cc.	Single injection	Intradermal	100 m.l.d.	0/3
	3	1.0 cc.	Two injections of 0.5 cc. each, spaced 1 week apart	Intradermal	100 m.l.d.	0/3
	2 (controls)	.....	.....	Intradermal	100 m.l.d.	0/2
Crystal violet-inactivated virus	4	2.5 cc.	Single injection	Subcutaneous	100 m.l.d.	0/4
	4	5.0 cc.	Two injections of 2.5 cc. each, spaced 1 week apart	Subcutaneous	100 m.l.d.	0/4
	2 (controls)	.....	.....	Intradermal	100 m.l.d.	0/2
Live virus	7	1.0 cc. (1,000 m.l.d.)	Single injection	Subcutaneous	.....	0/7
	2 (controls)	.....	.....	Intradermal	100 m.l.d.	0/2

*Challenge virus administered twenty-four hours following vaccination.

of each drug are shown in table 2. It was planned to administer the chloromycetin by the intravenous route. However, such severe tissue damage resuled from the initial inoculation that all injections were made subcutaneously thereafter. For the same reason, the terramycin also was given subcutaneously. The course of the infection in the treated and control animals was followed both by daily temperature recordings and by clinical observations. Blood-drug levels were determined in the case of all but the chloromycetin-treated animals using a tube-dilution method and a suitable test organism. Because of the hazard involved in making repeated heart punctures, assays were conducted on the serums of 2 animals only from each group, drawn at intervals following the injection of the drugs on the first and second days of treatment. Treatment was carried out for seven days or for such lesser time as the rabbits survived.

RESULTS

As shown in table 2, none of the antibiotics employed exerted any effect on the course of the disease.

DISCUSSION

The results of the investigation reported herein strongly suggest that, inasmuch as failure was experienced in attempts to immunize rabbits against the California strain of myxoma virus by the use of tissues containing a high content of virus, there is little likelihood that successful immunization against this disease can be achieved by the use of inactivated vaccines. Since this disease apparently falls into the category of those against which little or no immunity can be produced by the inoculation of inactivated virus, the main hope of achieving successful artificial immunity would seem to be in the use of the active attenuated agent.

While some investigators have succeeded in protecting a certain percentage of animals against the California and South American strains of the virus by the use of inactivated vaccines prepared from blood and tissues of infected rabbits, these animals were subjected to a much less severe challenge than was employed in this study. However, it was considered that unless a vaccine could be developed which would stimulate, in all cases, an immune response capable of withstanding a reasonably strong challenge, it would be of little practical value.

On the basis of results obtained in the treatment of infectious myxomatosis with aureomycin, penicillin, chloromycetin, and terramycin, indications are that such agents are of no value in this disease. This finding is in keeping with the fact that thus far no antibiotic substances have been discovered which are effective against viruses of the same order of size as that of the myxoma virus.

SUMMARY

Attempts to immunize domestic rabbits against infectious myxomatosis by means

TABLE 2—Effect of Antibiotic Therapy on Rabbits Experimentally Infected with Myxoma Virus

Antibiotic	No. rabbits used	Dosage and treatment schedule	Route of injection	No. days treated	Average blood-drug level	Survival ratio
Aureomycin	8	Initial dose: 5 mg./lb. body wt. once daily; thereafter, 2.5 mg./lb. body wt. once daily	Subcutaneous	5-7	3.18 μg./cc. blood serum	0/8
.....	2 (controls)	.....	.....	...	.....	0/2
Penicillin	6	100,000 units once daily	Intramuscular	5-7	0.90 units/cc. blood serum	0/6
.....	1 (control)	.....	.....	...	.....	0/1
Chloromycetin	6	100 mg./kg. body wt. in divided doses at 9 a.m., 3 p.m., and 9 p.m. daily	Subcutaneous	5-7	Determinations not made	0/6
.....	6 (controls)	.....	.....	...	.....	0/6
Terramycin	6	25 mg./kg. body wt. in equally divided doses daily at intervals of 12 hours	Subcutaneous	5	1.0 μg./cc. blood serum	0/6
.....	4 (controls)	.....	.....	...	.....	0/4

of heat and chemically inactivated, egg-adapted myxoma virus were unsuccessful.

Aureomycin, penicillin, chloromycetin, and terramycin were found to be of no therapeutic value in this disease.

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A Key For Tentative Recognition of Bacteria Commonly Encountered in Clinical Bacteriology

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THE IDENTIFICATION of microörganisms encountered in clinical specimens appears to many laboratory workers to be an involved and laborious task, unless the cultures examined happen to be members of groups well known to the worker. This situation arises, largely, from the fact that "Bergey's Manual of Determinative Bacteriology"[1] lists some sixteen hundred species of microörganisms in 152 genera and subgenera, and the investigator is sometimes uncertain as to the best way to determine the genus to which a somewhat unfamiliar organism belongs.

It was to alleviate this condition that the accompanying key for identification to genus was devised. Its simplicity rests upon two facts: first, the number of genera commonly encountered in clinical specimens either as primary or secondary invaders is not large; and second, the number of essential characteristics which serve to distingish one genus from another within this group is not great. The many charactertistics which vary within different genera need not be considered when genus identification is desired. Once tentative identification to genus is accomplished, confirmation of this and identification to species are usually swiftly and surely accomplished.

This key has served satisfactorily in the hands of the author for several years, and also in the hands of students. It is based, largely, on "Bergey's Manual of Determinative Bacteriology," the mechanical key for generic identification of Skerman,[2] and on publications restricted to certain genera. The great majority of genera listed in Bergey's Manual are not to be found in the accompanying key, for they are encountered so infrequently in clinical specimens that they may be disregarded. Consequently, those bacteria which are plant pathogens, fresh or salt water forms, or which live in some special habitat or require very special mediums or conditions for growth have been omitted. Some microörganisms, such as Streptomyces, which are nonpathogenic have been included, since they appear not uncommonly as contaminants or as secondary invaders and their recognition is convenient.

Organisms should be studied more thoroughly than is indicated by this key. The characters used for tentative identification are quite reliable, but should be supplemented by determination of serological, pathogenic, toxicogenic, or biochemical characteristics, depending upon the genus concerned.

GRAM-POSITIVE RODS, NOT FORMING MYCELIUM, NO BRANCHING[a]

- Acid-fast—Mycobacterium
- Not acid-fast
  - Forming spores
    - Catalase[b]-positive—Bacillus
    - Catalase-negative—Clostridium
  - Not forming spores
    - Catalase-positive
      - Nonmotile[c]—Corynebacterium
      - Motile—Listeria
    - Catalase-negative
      - Good growth in litmus milk with definite acid production or coagulation—Lactobacillus
      - Usually no growth in litmus milk; if so, slight if any acid production—Erysipelothrix

GRAM-POSITIVE RODS, FORMING MYCELIA WITH TRUE BRANCHING IN YOUNG CULTURES

- Aërobic
  - No true conidia, often acid-fast—Nocardia
  - True conidia formed—Streptomyces
- Anaërobic—Actinomyces

GRAM-NEGATIVE RODS, RIGID CELLS

- Aërobic
  - Spiral cells—Spirillum[d]
  - Bent cells—Vibrio[d]
  - Straight cells
    - Forming water-soluble pigment—Pseudomonas
    - No water-soluble pigment formed
      - Grow well on beef extract, peptone agar
        - Lactose fermented rapidly

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Methyl red positive, VP negative—Escherichia$_a$
Methyl red negative, VP positive—Aerobacter$_a$
Heavily encapsulated, methyl red variable—Klebsiella
Lactose not fermented
Urease$_f$-positive—Proteus
Urease-negative
Glucose fermented with production of gas, usually motile—Salmonella$_g$
Glucose fermented with production of acid only, nonmotile—Shigella$_g$
Glucose not fermented—Alcaligenes
Lactose fermented slowly (2-30 days)—paracolons, also *Shigella sonnei, Shigella equirulis, and Shigella dispar*
Grow poorly with small colonies or not at all on beef extract, peptone agar; cells small
Usually show bipolar staining, ferment carbohydrates
Colonies usually adherent to agar, milk unchanged to slightly acid—Actinobacillus
Colonies not adherent
Milk not coagulated—Pasteurella
Milk coagulated slowly or digested—Malleomyces
Usually no bipolar staining, carbohydrates not fermented, urease-positive—Brucella
Grow not at all on extract agar and require, especially on first isolation, either hematin (X factor) or diphosphopyridine nucleotide (V factor), or both
Nonmotile
Acid from glucose, usually single cells—Hemophilus
No acid from glucose, usually diplobacilli—Moraxella
Motile, often encapsulated—Noguchia

Anaërobic$_h$
Pleomorphic cells with rounded ends—Bacteroides
(This includes *Spherophorus necrophorus*, Ristella, etc.)
Cells with tapered ends, 3 to 16 μ long—Fusobacterium (This includes Fusiformis)

GRAM-NEGATIVE RODS, CELLS FLEXUOUS, NOT RIGID

Stain easily with aniline dyes—Borrelia
Stain with difficulty except for Giemsa's, Wright's, or silver-impregnation stains
Aërobic—Leptospira
Anaërobic—Treponema

GRAM-POSITIVE COCCI

Catalase-positive, usually in tetrads—Gaffkya; usually in irregular masses—Micrococcus
Catalase-negative, usually in pairs or chains
Soluble in bile, cells usually in pairs—Diplococcus
(Anaërobic species of this genus are not bile-soluble, but are rarely encountered.)
Not soluble in bile, cells usually in chains
No gas from glucose—Streptococcus
Gas from glucose—Leuconostoc

GRAM-NEGATIVE COCCI

Usually occurring in pairs—Neisseria
Usually occurring in masses—Veillonella

NOTES

a) Rudimentary branching is not uncommon in the genera Mycobacterium and Corynebacterium, and has been observed in other genera. However, it is generally so rare elsewhere and so marked at some stages of growth in Nocardia, Streptomyces, and Actinomyces that confusion on this point is unlikely.

b) Catalase tests are most easily done by removing a portion of the colony and suspending it in a drop of 3 per cent hydrogen peroxide. The formation of bubbles of oxygen indicates the production of catalase by the organism concerned. Care should be exercised if this test is used with colonies grown on blood agar, for the red cells of all species of animals contain catalase; consequently one should make certain that a positive catalase reaction is not due to catalase from the blood agar.

c) Motility is best determined by the use of semisolid "motility" agar, particularly when sluggishly motile species are concerned.[3] Microscopic observations of motility are reliable when motility is definitely observed; when it is not observed, the use of semisolid medium is advisable.

d) The distinction between these two groups may sometimes be difficult, for some organisms which are definitely Vibrio-like during early stages of growth later become just as definitely Spirillum-like.

e) A number of forms intermediate between these two genera are known. Few, if any of them, are pathogenic.

f) The rapid urease test of Stuart *et al.*[4] is convenient here.

g) Cultures falling into these genera should be checked for the biochemical characteristics suggested by Edwards and Ewing.[5] It should be noted that *Salmonella typhosa* and *Salmonella gallinarum* do not produce gas from glucose and that *Salmonella pullorum* and *S. gallinarum* are nonmotile.

h) Classification of the nonspore-forming anaërobic bacteria is, at present, unsatisfactory. See appendixes to Bacteriodes and Fusobacterium in Bergey's Manual[1] or Prevot.[6]

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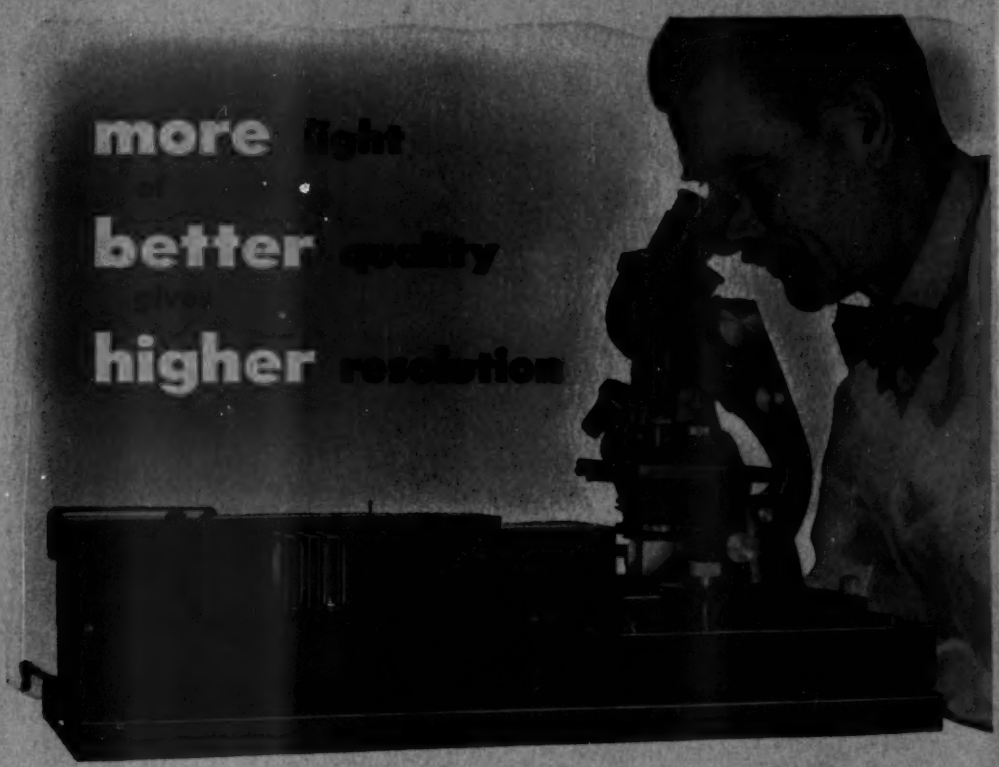

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